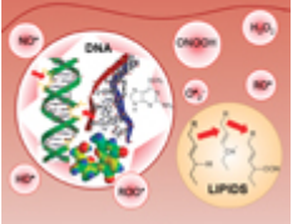
ACS SYMPOSIUM SERIES 1083

Oxidative Stress: Diagnostics, Prevention, and Therapy


NO•
DNA
ONOO⁻
H₂O₂
RO•
O₂•
HO•
ROO•
LIPIDS

EDITED BY

Silvana Andreescu and Maria Hepel

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.fw001

Oxidative Stress: Diagnostics, Prevention, and Therapy

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.fw001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.fw001

ACS SYMPOSIUM SERIES **1083**

Oxidative Stress: Diagnostics, Prevention, and Therapy

Silvana Andreescu, Editor
Clarkson University
Potsdam, New York

Maria Hepel, Editor
State University of New York at Potsdam
Potsdam, New York

Sponsored by the
ACS Division of Analytical Chemistry

American Chemical Society, Washington, DC

Distributed in print by Oxford University Press, Inc.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.fw001

Library of Congress Cataloging-in-Publication Data

Oxidative stress : diagnostics, prevention, and therapy / Silvana Andreescu, Maria Hepel, editor[s] ; sponsored by the ACS Division of Analytical Chemistry.
p. cm. -- (ACS symposium series ; 1083)
Includes bibliographical references and index.
ISBN 978-0-8412-2683-8 (acid-free paper) 1. Oxidative stress--Physiological effect. 2. Antioxidants. I. Andreescu, Silvana. II. Hepel, Maria. III. American Chemical Society. Division of Analytical Chemistry.
RB170.O943 2011
616.3′9--dc23
2011042499

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48n1984.

Copyright © 2011 American Chemical Society

Distributed in print by Oxford University Press, Inc.

All Rights Reserved. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Act is allowed for internal use only, provided that a per-chapter fee of $40.25 plus $0.75 per page is paid to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. Republication or reproduction for sale of pages in this book is permitted only under license from ACS. Direct these and other permission requests to ACS Copyright Office, Publications Division, 1155 16th Street, N.W., Washington, DC 20036.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.fw001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.pr001

Preface

Oxidative stress is a state where a biological system has a diminished capacity to counteract an overproduction or invasion of reactive oxygen species and other radicals. This book presents recent achievements in the understanding of oxidative stress, the mechanisms of damage to cells, DNA and proteins, and involvement in premature aging and major diseases, including cancer, Alzheimer's, autism, HIV infection and stroke, among others. The book is a comprehensive source of information providing some of the latest directions in diagnostics of oxidative stress, including novel methods for detecting oxidative-stress biomarkers, prevention by antioxidants, and therapy using newly developed free radical-scavenging agents and natural defense systems.

The book covers topics beginning from general effects of oxidative stress on human health and the role of antioxidants, through the important problems of disease prevention and treatment, including mechanisms of action of naturally occurring antioxidants, systemic defenses and redox homeostasis, artificial nanoparticle antioxidants and treatment of neurological diseases associated with oxidative stress, and finishing with examples of modern analytical platforms for the detection of free radicals and oxidative stress metabolites, including electrochemical sensors and biosensors and mass spectrometry approaches. Several chapters present reviews on important subjects of oxidative stress and implications of oxidative stress in disease progression and therapy, mechanisms of oxidative damage as well as antioxidants and prevention by antioxidants. Other chapters describe analytical methodologies for the detection of free radicals and protein interactions.

This work showcases new investigations and discoveries made in research groups around the world. We hope that this book will provide for a novice a solid background on oxidative stress and its consequences, methods of detection, disease prevention and therapy. For scientists and practitioners already involved in this field we aim to provide a wealth of information on the most recent achievements and new insights to the broad subject matter of oxidative stress, e.g. detection of biomarkers, mutagenesis and progressive damage to DNA, proteins and lipids, mechanism of free radical reactions, redox regulation in biological systems, and novel antioxidant therapies for the treatment of oxidative stress diseases. Since this field is rapidly expanding, with new discoveries of implications of oxidative stress in many common diseases being reported every year, we hope that a wide audience will find this book useful as a database and a collection of concepts and ideas to build on in further studies.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Silvana Andreescu
Clarkson University
Potsdam, NY

Maria Hepel
State University of New York at Potsdam
Potsdam, NY

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.pr001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Chapter 1

The Role of Antioxidants in Human Health

Saikat Sen* and Raja Chakraborty

Creative Educational Society's College of Pharmacy, Kurnool, Andhra Pradesh -518 218, India
***E-mail: saikat.pharm@rediffmail.con. Phone: +91-9032011182.**

Recent development in medicinal field reports a number of disease associated with free radicals. The risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure of chemicals, pollution, cigarette smoking, drugs, illness, and stress etc. Antioxidants are the substances which can scavenge free radicals and help to decrease the incidence of oxidative stress induced damage. Traditional herbal medicines, dietary foods were the main source of antioxidant for ancient peoples that protected them from the damage caused by free radicals. Over consumption of antioxidants however can cause severe adverse reactions as well. In this chapter we have discuss the concept of free radical biology and role of different antioxidants on human health and diseases.

Introduction

Oxygen is an indispensable element for life. Oxidative property of oxygen plays a vital role in various biological phenomena; being essential for life, oxygen can also aggravate the damage within the cell by oxidative events. Oxygen is used by the cell to generate energy and free radicals are formed as a consequence of ATP (adenosine triphosphate) production by the mitochondria. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products resulting from the cellular redox process. These reactive species play a dual role in human as both toxic and beneficial compounds. The delicate balance between their two opposite effects is undoubtedly a key aspect of life. At low or moderate levels, reactive species exert beneficial effects on cellular redox signalling and immune function, but at high concentrations, they produce oxidative stress, a harmful process that can damage cell function and structures (*1*, *2*).

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

The causes of the toxic properties of oxygen were obscure prior to Gershman's free radical theory. World War II (1939-1945) gave rise to the field of free radical biochemistry. Gershman and Gilbert speculated that the lethal and toxic effects of ionizing radiation following the aftermath of Hiroshima and Nagasaki might be due to cellular action of ROS. In 1954 Gershman's free radical theory stated that the toxicity of oxygen is due to partially reduced forms of oxygen. In the same year, Commoner, Townsend, and Pake reported a weak electron paramagnetic resonance (EPR) signal attributable to the presence of free radicals in a variety of lyophilised biological materials. Soon thereafter Denham Harman (1956) explored the world of free radicals in biological systems and proposed the concept of free radicals playing a role in the ageing process. These early studies triggered intense research in the area of free radicals in biological systems for decades to come (*3–5*).

Free radicals induced oxidative stress is now believed to be a fundamental mechanism underlying a number of human cardiovascular, neurologic and other disorders. Antioxidants are our crucial defense against free radical induced damage, and are critical for maintaining optimum health and wellbeing. It has been estimated that ~ 5% of inhaled oxygen is converted into several damaging ROS species like superoxide, hydroxyl and hydrogen peroxide by equivalent reduction of oxygen. In today's modern world the risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure of chemicals, pollution, cigarette smoking, drugs, illness, and stress etc. Exogenous consumption of antioxidants from plant, animal, and mineral sources have proved beneficial to human health and effective to reduce the incidence of free radical induced diseases. The antioxidants are also associated with reduction of free radical generation and improves antioxidant status in patients, thus it may be beneficial to recover normal function and treatment of such diseases. In recent years there has been increased interest in the therapeutic use of antioxidants in the treatment of disease associated with oxidative stress (*6–8*). Several studies reported that low antioxidant intake or low blood levels of antioxidants increases the risk of different diseases, infect low dietary intake of fruits and vegetables doubles the risk of cancer (*7*). Therefore, wholesome antioxidant diet and natural antioxidant supplements as part of a healthy lifestyle are now being recognized to protect health from oxidative stress.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Concept of Free Radical Biology

ROS and RNS are the by-products result from the cellular redox process and are well documented for playing a dual role as both deleterious and beneficial species. Though in normal physiological condition the generation of reactive species are tightly regulated by different enzymatic and non-enzymatic antioxidant, but overproduction of ROS results in oxidative stress, which is important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA (*4*, *9*).

Free Radicals and Their Sources

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in the outer orbit. This unpaired electron(s) are unstable and usually gives a significant degree of reactivity to the free radical. ROS includes superoxide ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals. Nitrogen free radicals includes nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). Oxygen and nitrogen free radicals can be readily converted to other non-radical reactive species which are also dangerous for health. Hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH) are not free radicals and generally named oxidants and can easily lead to free radical reactions in living organisms. Oxidants are also capable of nirosylating proteins thereby disrupting biological function. Thus, ROS and RNS include radical and non-radical species. These reactive species are produced in animals and humans under physiologic and pathologic conditions (*1*, *4*, *9*, *10*).

Free radicals can be produced from both endogenous and exogenous substances. Production of free radicals in the body is continuous and a normal part of our physiology. The biological process associated with free radical generations includes the following (*1*, *2*, *11*, *12*).

- *Immune system*: Immune system cells generate oxy-radicals and ROS in response to pathogens.
- *Metabolic process*: Free radicals can generate during metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells. Lipid peroxidation an important source of free radicals and can formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidases, neutrophils. Mitochondria generate continuously and abundantly oxy-radicals and ROS as toxic waste through a number of metabolic processes, each of which can produce different free radicals.
- *Inflammation*: Inflammation releases cytokines and initiates neutrophils and macrophages to produce free radicals.
- *Stress*: Mental and body's stress can trigger the production of free radicals as a toxic by-product. Additionally, the hormones that mediate the stress reaction in the body like cortisol and catecholamine themselves degenerate into destructive free radicals.
- *Pollution*: The different type of pollutants like air pollutants (asbestos, benzene, carbon monoxide, chlorine, formaldehyde, ozone, and toluene), chemical solvents (cleaning products, glue, paints, paint thinners, perfumes, and pesticides), and water pollutants (chloroform and other trihalomethanes) are all potent generator of free radicals. Burning of organic matter during cooking, forest fires, and volcanic activities also can generate free radicals.
- *Radiation*: UV radiations, medical and dental x-rays, gamma rays, and microwave radiation can lead to free radical generation.
- *Dietary factors*: Additives, alcohol, coffee, foods from animal origin, foods that have been barbecued, broiled fried, grilled, or otherwise

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

cooked at high, temperatures, foods that have been browned or burned, herbicides, hydrogenated vegetable oils, pesticides, sugar and processed foods containing high levels of lipid peroxides, and can produce free radicals.

- *Toxins and drugs*: Carbon tetrachloride, paraquat, benzo pyrene, aniline dyes, toluene and drugs like adriamycin, bleomycin, mitomycin C, nitrofurantoin, chlorpromazine etc. increases free radical productions.
- *Other factors*: Automobile exhausts fumes, smoking of tobacco products, cause free radicals generation.

Biological Role of Free Radicals

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

The beneficial effects of ROS and RNS occur at low/moderate concentrations and involve in several normal physiological function against several cellular responses. Most cells can produce superoxide, hydrogen peroxide and nitric oxide constitutively while others have inducible ROS/RNS release system. For example defense against infectious agents by phagocytosis, killing of cancer cells by macrophages and cytotoxic lymphocytes, detoxification of xenobiotics by Cytochrome P450, generation of ATP in mitochondria (energy production), cell growth, and the induction of mitogenic responses at low concentrations are some key beneficial activities of ROS and RNS. They also play important role on different cellular signalling at low concentration like activation of several cytokines and growth factor signalling, non-receptor tyrosine kinases activation, protein tyrosine phosphatises activation, release of calcium from intracellular stores, activation of nuclear transcription factors. ROS exert vital actions such as gene transcription, and regulation of soluble guanylate cyclase activity in cells. NO produced by endothelial cells is essential for blood pressure regulation of vascular smooth muscle, leukocyte adhesion, platelet aggregation, angiogenesis, and thrombosis. In addition, NO produced by neurons is an important neurotransmitter and key for neural plasticity, and NO generated by activated macrophages is a key mediator of the immune response. Moreover, recent studies also suggest that the ROS, such as superoxide, hydrogen peroxide may act as second messengers, but may be harmful as the accumulation of these free radicals are increased (*3*, *4*, *9*, *13*).

Scavenging of Free Radicals

Free radicals derived from radicals in presence of oxygen and function at low, but measurable concentrations in the cells. The “steady state” concentrations of free radicals are determined by the balance between their rates of production and their rates of removal by various antioxidants. Thus the redox state of a cell and its oscillation determines cellular functioning. Ironically, different ROS-mediated activities in fact protect cells against ROS-induced damage and re-establish or maintain “redox balance” termed also “redox homeostasis”. But the ROS in higher concentration responsible for cellular damage, therefore, to protect the cells and organ systems of the body against free radicals, humans have evolved an extremely sophisticated and complex antioxidant protection system. Antioxidants are defined as the substance which at low concentration significantly inhibit or delay the oxidative process, while often being oxidized themselves. Endogenous and exogenous antioxidants are used to neutralize free radicals and protect the body from free radicals by maintaining radox balance (*1*, *4*, *10*, *14*, *15*). These antioxidants can classify into different groups. A detailed classification antioxidants are given in Table 1.

Superoxide dismutase (SOD) is an important endogenous antioxidant enzyme and can exist in several common forms. They are proteins contain copper and zinc, or manganese, iron, or nickel and act as the first line defense system against ROS which scavenges superoxide radicals. SOD catalyzes the dismutation of $O^{2\bullet-}$ to H_2O_2 and O_2. Humans contain three forms of superoxide dismutase: SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. Catalase (CAT) is a common, most efficient enzyme found cell. It is a tetramer of four polypeptide chains and contains four porphyrin heme groups which allow the enzyme to react with the hydrogen peroxide. Each catalase molecule can decompose millions of hydrogen peroxide molecules to water and oxygen in every second. Glutathione peroxidase (GPx) present in the cytoplasm of the cells and protect the cell against oxidative injury caused by H_2O_2 and prevent the formation of hydroxyl radical from H_2O_2. It consists of four protein subunits; each of which contains one atom of the element selenium at its active site. GPx remove H_2O_2 by coupling its reduction to H_2O with oxidation of GSH. Glutathione reductase (GR) is flavoprotein enzyme and important cellular antioxidant necessary for the conversion of GSH. The oxidised glutathione is glutathione disulphide (GSSG) which reduced back to GSH by the presence of enzyme GR which uses NADPH as an electron donor. The ratio of GSH/GSSG is an important general measure of oxidative stress of an organism, very high concentration of GSSG may damage many enzymes oxidatively (*4*, *9*, *10*).

Glutathione is a tripeptide and a powerful antioxidant highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble, non-enzymatic antioxidant in these cell compartments. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are essential for DNA repair and expression. It is the major intracellular nonprotein thiol compound (NPSH) synthesized intracellularly from cysteine, glycine and glutamate. GSH is important in maintaining -SH groups in other molecules including proteins, regulating thiol-disulfide status of the cell, and detoxifying foreign compounds and free radicals. Sulphur containing amino acids like methionine and cysteine are precursors of GSH but also provide -SH groups to react with H_2O_2 and the $OH^\bullet$ radical and may prevent tissue damage. GSH is capable of scavenging hydroxyl radical and singlet oxygen directly, or detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of GPx. GSH also involved in amino acid transport through the plasma membrane, regeneration of some important antioxidants, vitamins C and vitamin E is regulate by the glutathione; for example glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate. Lipoic acid is another endogenous antioxidant, characterized as a “thiol” or “biothiol,” is a sulfur-containing molecule. Lipoic acid and its reduced form, dihydrolipoic acid has been called a “universal antioxidant, as these are

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

capable of quenching free radicals in both lipid and aqueous domains. Lipoic acid may also produce its antioxidant effect by chelating with pro-oxidant metals (*4*, *7*, *9*, *10*, *16*). Exogenous antioxidants are also play vital role to help the endogenous antioxidant to protect the body. Pathways of ROS/RNS formation, lipid peroxidation process and role of different antioxidants are given in Figure 1.

Table 1. Classification of antioxidants

A. CLASSIFICATION BASED UPON THEIR NATURE

1. **Enzymatic antioxidant**

   Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and Glutathione reductase (GR).

2. **Non-enzymatic antioxidant**

   a) ***Metabolic antioxidants***

   Reduced glutathione (GSH), lipoid acid, L-arginine, coenzyme Q_{10}, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc

   b) ***Nutrient antioxidants***

   Vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc

B. CLASSIFICATION BASED UPON SOURCE

1. **Endogenous Antioxidants**

   Bilirubin, glutathione, lipoic acid, *N*-acetyl cysteine, NADPH and NADH, ubiquinone (coenzyme Q_{10}), uric acid, enzymes (SOD, CAT, GPx, GR).

2. **Dietary Antioxidants**

   Vitamin C, Vitamin E, Beta carotene and other carotenoids and oxycarotenoids (lycopene and lutein), polyphenols (flavonoids, flavones, flavonols, and proanthocyanidins

3. **Metal Binding Proteins**

   Albumin (copper), ceruloplasmin (copper), metallothionein (copper), ferritin (iron), myoglobin (iron), transferrin (iron).

C. CLASSIFICATION BASED MECHANISM OF ACTION

1. **Catalytic systems to neutralise or divert ROS**

   SOD, CAT, GPx

2. **Binding/inactivation of metal ions prevents production of ROS by Haber–Weiss reaction**

   Ferritin, caeruloplasmin, catechins

3. **Self suicidal and chain breaking antioxidants scavenge, destroy ROS**

   Vitamin C, vitamin E, uric acid, glutathione, flavonoids

4. **Quenching ROS, chemical traps/sinks to 'absorb' energy**

   Carotenoids, anthocyanidins

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

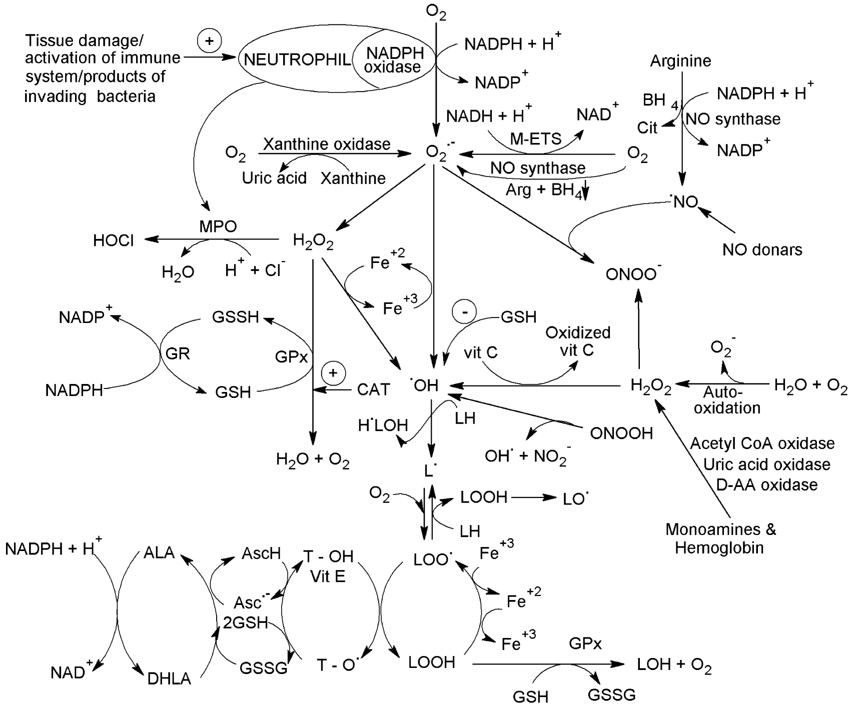

O_2
Tissue damage/ activation of immune system/products of invading bacteria
+
NEUTROPHIL
NADPH oxidase
NADPH + H^+
$NADP^+$
Arginine
BH_4
NADPH + H^+
NO synthase
Cit
$NADP^+$
NADH + H^+
NAD^+
M-ETS
O_2
Xanthine oxidase
$O_2^{\bullet-}$
O_2
Uric acid
Xanthine
NO synthase
Arg + BH_4
$^{\bullet}NO$
NO donars
MPO
HOCl
H_2O_2
H_2O
$H^+ + Cl^-$
Fe^{+2}
Fe^{+3}
$ONOO^-$
$NADP^+$
GSSH
GR
GPx
NADPH
GSH
-
GSH
vit C
Oxidized vit C
+
CAT
$^{\bullet}OH$
H_2O_2
O_2^-
$H_2O + O_2$
Auto-oxidation
$H^{\bullet}LOH$
LH
ONOOH
$H_2O + O_2$
$L^{\bullet}$
$OH^{\bullet} + NO_2^-$
Acetyl CoA oxidase
Uric acid oxidase
D-AA oxidase
O_2
LOOH
$LO^{\bullet}$
LH
Monoamines & Hemoglobin
NADPH + H^+
ALA
AscH
T - OH
Vit E
$LOO^{\bullet}$
Fe^{+3}
$Asc^{\bullet-}$
2GSH
Fe^{+2}
Fe^{+3}
GPx
NAD^+
DHLA
GSSG
T - $O^{\bullet}$
LOOH
LOH + O_2
GSH
GSSG

Figure 1. Pathway of ROS, RNS formation, the lipid peroxidation process and role of several antioxidants in the management of oxidative stress. O_2, oxygen; NO, nitric oxide; •NO, nitric oxide radical; M-ETS, mitochondrial electron transport system; BH_4, (6R)-5,6,7,8,-tetrahydro-L-biopterin; AA, amino acid; Arg, L-arginine; Cit, L-citrulline; $ONOO^-$, peroxynitrite; HOCl, hypochlorous acid; MPO, myeloperoxidase; H_2O_2, hydrogen peroxide; H_2O, water; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); AA, amino acid; L•, lipid radical; LH, lipid (unsaturated fatty acid); H•LOH, hydroxy lipid radical; LO•, lipid alkoxyl radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; LOH, form of alcohol; Vit C, vitamin C; Vit E, vitamin E; GSH, glutathione; GSSH, oxidised glutathione; GR, glutathione reductase, GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; $O_2^{\bullet-}$, superoxide radical; •OH, hydroxyl radical; T-O•, Vitamin E radical; T-OH, Vitamin E; $Asc^{\bullet-}$, ascorbyl radical; AscH, ascorbate monoanion; DHLA, dihydrolipoic acid; ALA, α-lipoic acid.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Concept of Oxidative Stress and Molecular Damage

The relation between free radicals and molecular damage can be described by the concept of ‘oxidative stress’. The harmful effect of free radicals causing potential biological injury is termed oxidative stress and nitrosative stress. The term oxidative stress has coined to describe a harmful condition caused by the excess of ROS production and/or a decrease in antioxidant levels. Similarly overproduction of reactive nitrogen species is named as nitrosative stress. Oxidative stress is the condition described as a shift towards the pro-oxidants in the pro-oxidant/antioxidant balance that can occur as a result of an increase in oxidative metabolism. Prolonged exposure to free radicals, even at a low concentration, may responsible for the damage of biologically important molecules and potentially lead to tissue injury. Oxygen is essential for the generation of all ROS and RNS. Thus, although molecular oxygen is absolutely essential for aerobic life, it can be toxic under certain conditions and this phenomenon has been termed the oxygen paradox (*3*, *9*, *15*, *17*).

Oxidative stress causes different diseases via four critical steps; membrane lipid peroxidation, protein oxidation, DNA damage and disturbance in reducing equivalents of the cell; which leads to cell destruction, altered signaling pathways. Oxidative stress has been implicated in various diseases like cancer, cardiovascular diseases, neurological disorders, diabetes, and ageing. Different mechanism are involved in the pathogenesis of such diseases such as “mitochondrial oxidative stress” conditions which can exemplify by pro-oxidants shifting the thiol/disulphide redox state and damaging glucose tolerance; “inflammatory oxidative conditions” and increased activity of either NADPH oxidase or xanthine oxidase-induced formation of ROS; or by both. Ageing is mainly due to the result of lipid peroxidation, DNA damage, protein oxidation by free radical action. Every biological molecules present in our body are at risk of damage by free radicals. Such damaged cell molecules can impair cell functions or can lead to cell death ultimately resulting in diseased states (*3*, *4*, *9*).

Lipids

Lipids present in membrane of subcellular organelles are highly susceptible to free radical damage. Free radical when reacted with lipid can undergo the highly damaging chain reaction of lipid peroxidation leading to both direct and indirect effects. Lipid peroxidation leads the generation of large number of toxic by-products that can have effects at a site away from the area of generation, act as ‘second messengers’. Lipid peroxidation induced damage is highly detrimental to the functioning of the cell. Lipid peroxidation in cell membranes can damage cell membranes by disrupting fluidity and permeability. Peroxidation of lipid is initiated by the attack of a species, which can remove a hydrogen atom from a methylene group, resulting the formation of an unpaired electron on the carbon atom ($^{\bullet}CH$). Carbon radical thus formed is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to form a lipid peroxyl radical ($LOO^{\bullet}$). These radicals can react with other lipid molecules to abstract hydrogen atoms further, so that lipid hydroperoxides (LOOH) forms and at the same time propagate other lipid peroxidation further (*3*, *4*, *9*, *11*, *18*).

Proteins

Proteins are also susceptible by the free radicals directly. Free radicals can cause the damage of many kind of protein, interfering with enzyme activity and function of structural protein. A highly reactive and stable product such as protein hydroperoxides can generate by the oxidation of proteins caused by ROS/RNS, which can generate additional radicals mainly upon interaction with transition metal ions. Although most oxidised proteins are functionally inactive in nature and are rapidly removed, but some can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases (*3*, *11*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

DNA

ROS/RNS interfere with DNA and leads to oxidative damage. DNA is highly susceptible to damage by the free radicals such as $^{\bullet}$OH. Theses can react with DNA by addition or loss of hydrogen atoms from the sugar moiety. In particular, the C4-C5 double bond of pyrimidine is very sensitive to attack by $^{\bullet}$OH, which results generation of a spectrum of oxidative pyrimidine damage products, such as thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Likewise, purines are susceptible to attach by $^{\bullet}$OH which leads to the generation of 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products. Free radical attack also causes the activation of the poly (ADP-ribose) synthetase enzyme which can leads to fragmentation of DNA and programmed cell death. This process depletes the cellular level of NAD+ levels thereby disrupting electron transport chain function (*3*, *9*, *11*, *19*).

Carbohydrates

Free radicals such as $^{\bullet}$OH react with carbohydrates by randomly resulting in carbon-cantered radical. This directs to chain breaks in vital molecules like hyaluronic acid (*3*).

In virtually free radical reactions have been implicated in every human pathological condition. Some of the human diseases where role of free radical well established includes neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, memory loss and depression), cardiovascular disease (atherosclerosis, ischemic heart disease, cardiac hypertrophy, hypertension, shock and trauma), pulmonary disorders (inflammatory lung diseases such as asthma and chronic obstructive pulmonary

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

disease), diseases associated with premature infants (bronchopulmonary, dysplasia, periventricular leukomalacia, intraventricular hemorrhage, retinopathy of prematurity and necrotizing enterocolitis), autoimmune diseases (rheumatoid arthritis), renal disorders (glomerulonephritis, tubulointerstitial nephritis, chronic renal failure, proteinuria, uremia), gastrointestinal diseases like peptic ulcer, inflammatory bowel disease and colitis), diabetes, tumours and cancers (*2*, *12*).

Antioxidants and Health

Antioxidants can produce their protective role against free radicals by as variety of different mechanism including (a) the catalytic systems to neutralise or divert ROS, (b) binding or inactivation of metal ions prevents generation of ROS by Haber-Weiss reaction, (c) suicidal and chain breaking antioxidants scavenge and destroy ROS, (d) absorb energy, electron & quenching of ROS. In 21st century, demand for intake of antioxidant food or dietary antioxidant increasing with the hope of keep body healthy and free from diseases (*20*, *21*).

Food and Antioxidants

Foods consumption is a major source of exogenous antioxidants and has been estimated that a typical diet provides more than 25,000 bioactive food constituents and many of this may modify a multitude of processes that are related to different diseases. Antioxidants are abundant in vegetables and fruits and are also found in grain cereals, teas, legumes, nuts and other food products. A systematic survey has identify more than 3100 antioxidant foods, beverages, spices, herbs and supplements which are regularly consumed by different cultures. Decrease in intake of nutritional and antioxidant food may increase the chance of oxidative stress which may leads to cell damage, therefore intake of such natural antioxidants may give protective effect against free radical induced diseases. A recent study reported that plant-based foods are generally higher in antioxidant content than animal-based and mixed food product. Beverages like unprocessed tea leaves, tea powders and coffee beans often have better antioxidant values from beer, wine and lemonades. The dairy products, meat, and fish generally have low in antioxidant content (*22*, *23*). Therefore herbs, spice, fruit and food from plant sources may still be important contributors to our antioxidant intake, especially in dietary cultures where spices and herbs are consumed regularly.

Dietary Antioxidant, Nutrient Antioxidant, and Antioxidant Supplements

The potential beneficial effects of antioxidants in protecting against disease have been well established. It is increasingly thought that nutrition may play a vital role in helping to defend against oxidative stress and damage induced by free radicals. Therefore, certain nutrients and dietary components with antioxidant properties are important for the protection against oxidative stress injury the body. In normal condition, our cell can capable to prevent free radical induced diseases by generation its own endogenous antioxidants or by take them from food (*24*,

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

25). Antioxidants through our diet play an important role in helping endogenous antioxidants for the neutralization of excess free radicals. Vitamin C, vitamin E, N-acetyl cysteine, carotenoids, co-enzyme Q_{10}, alpha-lipoic acid, beta carotene, lycopene, selenium, flavonoids etc are popularly used as dietary and nutritive antioxidant (*7*, *24*, *25*).

Vitamins C and vitamin E are the important non enzymatic antioxidant, which react with free radicals to form radicals themselves which are less reactive than the radicals. They break radical chain reactions by trapping peroxyl and other reactive radicals. Vitamin E is a key lipid-soluble antioxidant and a most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin E can transfer its phenolic hydrogen to a peroxyl free radical of a peroxidized PUFA (ω-6 polyunsaturated fatty acid), thus it break the radical chain reaction and prevent the peroxidation of PUFA in cellular and subcellular membrane phospholipids. Vitamin E is a chiral compound with eight stereoisomers (α, β, γ, δ tocopherol and α, β, γ, δ tocotrienol) but only α-tocopherol is the most bioactive form in humans. The important effect of vitamin E includes protection against colon, prostate and breast cancers, some cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders. Vitamin C is crucial for collagen, carnitine and neurotransmitters biosynthesis. Vitamin C act as reducing agent reacts with vitamin E radical to yield a vitamin C radical while regenerating vitamin E. Like a vitamin E radical, vitamin C radical is not a reactive species as its unpaired electron is energetically stable. It is possible that these vitamins are consumed in the process of lipid peroxidation induced by oxygen radicals in ischemia reperfusion to prevent the tissue damage. Health benefits of vitamin C include anti-atherogenic, anti-carcinogenic, immunomodulator, it is also beneficial in reducing the incidence of stomach cancer, in preventing lung and colorectal cancer (*1*, *4*, *9*, *16*).

β-carotene, is a fat soluble carotenoids which are considered pro-vitamins as they can be convert into active vitamin A (ratinol), which is essential for vision. It is consider as a strong antioxidant and the best quencher of singlet oxygen. It is provide antioxidant protection to lipid-rich tissues. Lycopene is another carotenoid with antioxidant activity. It found beneficial against prostate cancer. Selenium is a trace mineral required to forms the active site of several antioxidant enzymes including glutathione peroxidase. At low dose, it is important for its antioxidant, anti-carcinogenic and immunomodulator activity. Flavonoids are polyphenolic compounds found in most plant with antioxidant activity. More than 4000 flavonoids have been identified and divided into different subdivision like flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanidins. Different flavanoids are reported to prevent or delay a number of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, stroke, Alzheimer‘s disease, inflammation, infection (*3*, *7*, *9*).

Antioxidants like anthocyanins, beta-carotene, catechins, coenzyme Q_{10}, flavonoids, lipoic acid, lutein, lycopene, selenium, and vitamins C and E are abundant in vegetables, fruits and are also found in grain cereals, teas, legumes, and nuts; and now many antioxidants are also available as dietary supplements. Antioxidant supplements are often synthetic, and some of these synthetic forms may not have the same effects on the body as natural antioxidants occurring in foods due to the extensive processing associated with manufacturing. Although antioxidant molecules often prevent oxidative damage in laboratory experiments, there is some debate as to whether consuming antioxidants (in food or supplement form) actually benefits health in all conditions and the requirement of antioxidants. There is limited scientific data to support the use of antioxidant supplements to prevent disease based on clinical trials in humans. Antioxidants in foods are generally considered safe, and studies of antioxidant supplements generally have not reported adverse effects. Therefore more research is needed to better understand the safety aspects of dietary supplementation and it is advisable that antioxidant supplements should not be used as a replacement for a healthful diet or conventional medical care, without physician consultation (*23*, *26*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Antioxidants in Traditional Medicinal Systems

Over the centuries humans have relied on plants for basic needs such as food, clothing, and shelter and medicine. Traditional medicines or folk medicines in worldwide are the synthesis of the therapeutic experience of generations of practicing physicians of indigenous systems of medicine. The Indian, Chinese, Egyptian, Greek, Roman and Syrian traditional medicine comprise very ancient and reputed medicinal systems used natural substances derived mainly from plants for treatment of different disease. From ancient time, knowingly or unknowingly people are taking different fruits, vegetable, food or medicine from plant, animal and mineral sources which contain antioxidant constituent and responsible for several health benefits. Traditional or complementary medicine treats the body as a holistic organism with dynamic processes that maintain the body balance and keep us healthy. On a global scale there is substantial and justifiable recognition of the many benefits that orthodox medicine. Many compounds from natural sources have been used as drugs, either in their original or semi-synthetic form. Plant derived constituents can also serve as drug precursors, drug prototypes, and pharmacological probes (*27–29*). In different traditional medicinal systems plants are the primary component to treat a diseases and subsequently so many plants, fruit, vegetable are used to keep the body healthy. In the modern age exploring the different pharmacological action of traditional medicine, isolation of phyto-constituents is the main research interest. Many research studies have identified antioxidant activity in a wide array of plants across many traditional medicinal systems. Table II listed some of the traditional medicinal plants, which have found beneficial in treatment/prevention of several diseases through their antioxidant mechanism (*3*, *6*, *30*, *50*).

These are only some of the examples of the plants possess antioxidant activity, a lot more plants have screened for their antioxidant activity and still a lot of plant need to be screen. Theses plants are used in different health care system to treat different diseases or they are used as vegetable, or different parts of the plants are edible as fruit for salad and used regularly by people.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table II. Traditional medicinal plants with antioxidant activity

Traditional medicinal system	*Plants with antioxidant activity*
Indian (*Unani* and *Ayurveda*)	*Aegle marmelos, Allium cepa, Allium sativum, Aloe vera, Amomum subulatum, Andrographis paniculata, Asparagus racemosus, Azadirachta indica, Bacopa monniera, Cinnamomum verum, Cinnamomum tamala, Curcuma longa, Emblica officinalis, Glycyrrhiza glabra, Hemidesmus indicus, Momordica charantia, Nigella sativa, Ocimum sanctum, Picrorrhiza kurroa, Plumbago zeylanica, Syzigium cumini, Terminalia bellarica, Tinospora cordifolia, Trigonella foenumgraecum, Withania somnifera, Zingiber officinalis, Terminalia arjuna, Curcuma domestica, Cuscuta reflexa, Daucus carota, Foeniculum vulgare, Mangifera indica, Momordica charantia, Psoralea corylifolia, Santalum album, Solanum nigrum, Swertia chirayita, Baccharis coridifolia, Bryonia alba, Cichorium intybu, Cinnamomum zeylanicum, Crithmum maritimum, Cynara scolymus, Emilia sonchifolia, Eucalyptus camaldulensis, Eucalyptus rostrata, Eucommia ulmoides, Lavandula angustifolia, Lycium barbarum, Melissa officinalis, Murraya koenigii, Myrica gale, Panax ginseng, Piper nigrum, Plantago asiatica, Prunus domestica, Rhazya stricta, Rosmarinus officinalis, Salvia officinalis, Salvia triloba, Solanum melongena, Solanum tuberosum, Syzygium caryophyllatum, Eugenia caryophyllus, Thymus zygis, Tinospora cordifolia, Uncaria tomentosa, Centella asiatica*
Africa	*Sacoglottis gabonensis, Mallotus oppositifolium, Gongronema latifolium, Trichilia roka, Cussona barteri, Glinus oppositifolius, Lannea vilutina, Tapinanthus globiferus, Balanites aegyptiaca, Bombax costatum, Boscia senegalensis, Entada Africana, Gynandropis gynandra, Hypaene thebaica, Leptadenia hastate, Sesbania pachycarpa, Thonningia sanguinea, Dorstenia psilurus, Dorstenia ciliate, Dorstenia mannii, Sutherlandia frutescens, Pelargonium reniforme, Olea europa, Myrothamnus flabellifolia, Rhoicissus digitata, Rhoicissus rhomboidea, Rhoicissus tridentate, Eucalyptus Camaldulensis, Cleome Arabica, Burkea Africana, Salvia tiliifolia, Chamaecrista mimosoides, Buddleja salviifolia, Schotia brachypetala*
China	*Paeonia suffruticosa, Ligustrum lucidum, Morus alba, Paeonia lactiflora, Anemarrhena asphodeloides, Rehmannia glutinosa, Angelica keiskei, Leonurus heterophyllus, Asarum heterotropoides, Phaseolus radiatus, Ligusticum sinense, Morus alba, Angelica anomala, Alpinia oxyphylla, Angelica sinensis, Eucommia ulmoides, Spirodela polyrrhiza, Albizzia julibrissin, Atractylodes macrocephala, Cuscuta chinensis, Saposhnikovia divaricata, Ophiopogon japonicus, Clematis chinensis, Trichosanthes kirilowii, Codonopsis pilosula, Platycodon grandiflorum, Prunus persica, Magnolia liliflora, Ampelopsis japonica, Anoectochilus formosanus, Asparagus cochinchinensis, Panax ginseng, Prunus japonica, Achyranthes bidentata, Achyranthes longifolia, Agrimonia pilosa, Artemisia anomala, Artemisia argyi, Biota orientalis, Bletilla striata, Boehmeria nivea, Caesalpinia sappan, Campsis grandiflora, Carthamus tinctorius, Celosia, Cephalanoplos segetum, Cirsium japonicum, Curcuma aromatica, Curcuma wenyujin, Curcuma zedoaria, Cyathula officinalis, Dalbergia odorifera, Ginkgo biloba, Ilex pubescens, Impatiens balsamina, Imperata cylindrica, Leonurus heterophyllus, Ligusticum chuanxiong,*

Continued on next page.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table II. (Continued). Traditional medicinal plants with antioxidant activity

Traditional medicinal system	*Plants with antioxidant activity*
	Lycopus lucidus, Millettia dielsiana, Momordica cochinchinensis, Panax notoginseng, Polygonum cuspidatum, Polygonum orientale, Prunus persica, Rosa chinensis, Rubia cordifolia, Salvia miltiorrhiza, Sanguisorba officinalis, Scutellaria harbata, Selaginella tamariscina, Sophora japonica, Sparganium stoloniferum, Bothriospermum chinense, Cordyceps militaris, Scleria laeviformis, Dryopteris wallichiana, Aeschynanthus mengxingensis, Gaura parviflora, Lysimachia clethroides, Lysimachia heterogenea, Nardostachys chinensis, Valeriana jatamansi
Korea	*Saxifraga stolonifera, Pteridium aquilinum, Hemerocallis minor, Plantago asiatica, Osmunda japonica, Portulaca oleracea, Synurus deltoides, Ligularia fischeri, Rumex acetosa, Polygonum aviculare, Symplocarpus renifolius, Chenopodium album, Euonymus alatus, Cedrela sinensis, Nelumbo nucifera, Eucommia ulmoides, Zanthoxylum piperitum, Cudrania tricuspidata, Houttuynia cordata, Ulmus pumila, Rubus coreanus, Pinus densiflora, Carthamus tinctorius, Glycine soja, Glycine max, Lithospermum erythrorhizon, Prunus buergeriana, Prunus davidiana, Prunus padus, Prunus pendula, Prunus sargentii, Prunus serrulata, Prunus yedoensis*
Thailand	*Arcangelisia flava, Coscinium blumeanum, Fibraurea tinctoria, Amomum testaceum, Anethum graveolens, Angelica dahurica, Angelica sinensis, Artemisia, Atractylodes lancea, Cuminum cyminum, Dracaena loureiri, Foeniculum vulgare, Kaempferia galangal, Lepidium sativum, Ligusticum sinense, Oliv cv chuanxiong, Mammea siamensis, Mesua ferrea, Mimusops elengi, Myristica fragrans, Nigella sativa, Syzygium aromaticum, Acacia pennata, Anethum graveolens, Cassia siamea, Coccinia grandis, Coriandrum spp., Diplazium esculentum, Eryngium foetidum, Garcinia cowa, Lasia spinosa, Limnophila aromatica, Momordica charantia, Morinda citrifolia, Ocimum americanum, Ocimum gratissimum, Polygonum odoratum, Sesbania grandiflora, Sesbania javanica, Spilanthes acmella, Tiliacora triandra*
Jordon	*Punica granatum, Quercus calliprinos, Quercus calliprinos, Cinchona ledgeriana, Juniperus communis, Salvia fruticosa, Crataegus azarolus, Crataegus azarolus, Varthemia iphionoides, Artemisia herba-alba, Thymus capitatus, Morus nigra, Arum palaestinum, Matricaria aurea, Artemisia judaica, Teucrium polium, Pinus halepenss, Sarcopoterium spinosum, Crataegus azarolus, Inula viscose, Achillea fragrantissima, Hipiscus sabdariffa, Rhus cariara, Laurus nubilis, Feoniculum vulgare, Ocimum basilicum, Eruca sativa, Tregonella foenum grasem, Neigella sativa, Petroselinum sativum, Cuminum cymnium, Rumex pulcher, Beta vulgaris, Brassica nigra, Calamintha incana, Verbina triphylla, Portulaca aleraceae, Vitis vinifera, Cichorium pumilum, Nasturtium officinale*

Continued on next page.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table II. (Continued). Traditional medicinal plants with antioxidant activity

Traditional medicinal system	*Plants with antioxidant activity*
North America	*Ceanothus coeruleus, Chrysanctinia mexicana, Colubrina greggii, Cyperus alternifolius, Heliotropium angiospermum, Phyla nodiflora, Schinus molle, Licania arborea, Ficus involuta, Bunchosia cannesens, Syderoxylon capiri, Sapium macrocarpum, Ficus cotinifolia, Annona squamosa, Vitex molli, Piper leucophyllum, Gliricidia sepium, Cacahuano Tecahuananche, Astianthus viminalis, Hamelia patens, Swietenia humilis, Stemmadenia bella, Rupechtia fusca, Bursera grandifolia, Ziziphus amole, Jacaratia mexicana, Gyrocarpus jathrophifolius, Pseudobombax ellipticum, Comocladia engleriana, Plumeria rubra Tamarindus indica, Lippia alba, Pimenta dioica, Rheedia aristata, Rhus hirta, Cornus stolonifera, Nuphar variegatum, Quercus alba, Corylus cornuta, Kalmia angustifolia, Quercus rubra, Juniperus communis, Picea mariana, Sorbus americana, Gaultheria procumbens, Tsuga Canadensis, Prunus serotina, Picea glauca, Ledum groenlandicum, Abies balsamea, Sassafras albidum, Juniperus virginiana, Thuja occidentalis, Solidago Canadensis, Populus balsamifera, Taxus Canadensis, Achillea millefolium, Verbascum thapsus, Acorus calamus, Aralia nudicaulis, Asarum canadense, Celastrus scandens, Populus tremuloides, Aralia racemosa roots, Dirca palustris, Arisaema triphyllum roots, Taraxacum officinalis, Smilacina racemosa, Heracleum lanatum*
Central & South America	*Amphipterygium adstringens, Cordia boissieri, Acalypha alopecuroidea, Anthemis nobilis, Ambrosia artemisiaefolia, Arctostaphylos uva-ursi, Calluna vulgaris, Cassia fistula, Crataegus mexicana, Chiranthodendron pentadactylon, Cinchona succirubra, Castela texana, Echium Sp., Arnica Montana, Cassia obovata, Citrus limon, Artemisia vulgaris, Ocimum basilicum, Psacalium decompositum, Prunella vulgaris, Schinus molle, Senecio formosus, Tanacetum parthenium, Tagetes lucida, Leucophyllum frutescens, Satureja douglasii, Haplopappus baylahuen, Aristolochia giberti, Cecropia pachystachya, Eugenia uniflora, Piper fulvescens, Schinus weinmannifolia and Schinus terebinthifolia, Gentianella nitida, Iryanthera lancifolia, Lepechinia meyenii, Oenothera multicaulis, Philodendron solimoesense, Tetracera volubili*
Europe	*Acorus calamus, Alisma plantago-aquatica, Allium ursinum, Cotinus coggygria, Angelica sylvestris, Anthriscus cerefolium, Anthriscus sylvestris, Carum carvi, Daucus, Eryngium campestre, Sanicula europaea, Achillea millefolium, Arctium lappa, Artemisia absinthium, Artemisia vulgaris, Bellis perennis, Bidens tripartite, Carlina acaulis, Carthamus tinctorius, Cichorium intybus, Cirsium arvense, Conyza Canadensis, Hieracium pilosella, Matricaria recutita, Onopordum acanthium, Solidago virgaurea, Taraxacum officinale, Tussilago farfara, Betula pendula, Alliaria petiolata, Capsella bursa-pastoris, Nasturtium officinale, Humulus lupulus, Sambucus nigra, Sambucus ebulus, Viburnum lantana, Viburnum opulus, Evonymus europaeus, Cornus mas, Corylus avellana, Juniperus communis, Hippophae rhamnoides, Elaeagnus angustifolia, Equisetum arvense, Calluna vulgaris, Vaccinium myrtillus, Anthyllis vulneraria, Genista tinctoria, Lotus corniculatus, Ononis spinosa, Trifolium arvense, Trifolium pratense, Trifolium repens, Quercus petraea, Quercus robur,*

Continued on next page.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table II. (Continued). Traditional medicinal plants with antioxidant activity

Traditional medicinal system	*Plants with antioxidant activity*
	Centaurium erythraea, Erodium cicutarium, Geranium macrorrhizum, Geranium robertianum, Achillea millefolium, Arctium lappa, Betula pendula, Calendula officinalis, Cichohrium intybus, Clinopodium vulgare, Crataegus monogyna, Glycyrrhiza glabra, Humulus lupulus, Hypericum perforatum, Laurus nobilis, Matricaria chamomilla, Melissa officinalis, Mentha piperita, Mentha spicata, Rubus idaeus, Salvia officinalis, Sideritis scardica, Taraxacum officinale, Thymus vulgaris, Tilia cordata, Trigonella foenum-graecum, Urtica dioica, Hypericum perforatum, Ballota nigra, Galeopsis tetrahit, Galeopsis speciosa, Glechoma hederacea, Lamium album, Lamium maculatum, Lamium puprpureum, Leonurus cardiaca, Lycopus europaeus, Marrubium vulgare, Mentha aquatica, Mentha longifolia, Mentha pulegium, Origanum vulgare, Prunella vulgaris, Teucrium chamaedrys, Thymus serpyllum, Viscum album, Lythrum salicaria, Fraxinus ornus, Ligustrum vulgare, Syringa vulgaris, Chamerion angustifolium, Epilobium parviflorum, Oenothera biennis, Euphrasia officinalis, Chelidonium majus, Phytolacca americana, Pinus sylvestris, Plantago major, Elymus repens, Rumex acetosa, Primula elatior, Agrimonia eupatoria, Alchemilla vulgaris, Filipendula ulmaria, Fragaria vesca, Geum urbanum, Potentilla anserine, Prunus spinosa, Rosa canina, Rubus fruticosus, Sorbus aucuparia, Galium aparine, Ruscus aculeatus, Populus nigra, Populus tremula, Salix caprea, Verbascum densiflorum, Verbascum phlomoides, Verbascum thapsus, Tilia platyphyllos, Tilia tomentosa, Valeriana officinalis, Verbena officinalis, Viola tricolour

Phyto-Constituents as Antioxidant

Phytochemicals or plant constituents are the major source of antioxidants. The majority of these phytochemicals are redox active molecules; hence they are active to maintain redox balance and therefore defined as antioxidants. The chemicals from plants are generally classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents comprise of the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc., whereas secondary constituents includes alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived from carbohydrates). Antioxidant phyto-constituents are the secondary constituents or metabolites found naturally in plants such as fruits and vegetable. Plants produce a extremely impressive array of antioxidant compounds such as carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate. These plant-based antioxidants are believed to have a better biological effect than the synthetic one, because phytoconstituents are a part of the physiological functions of living flora and hence they are believed to have better compatibility with human body. Recent researches have shown that that plant extracts, phytoconstituents and food from plant sources have effective inhibitors of lipid peroxidation and oxidative stress. Antioxidants of plant origin with free-radical scavenging properties could have great importance as prophylactic and therapeutic agents in several diseases caused due to oxidative stress (*2*, *51*).

Synthetic Antioxidants

Chemically synthesized antioxidants are not in nature and often added to food as preservative to prevent lipid peroxidation. These antioxidants act mainly via two different pathways and known as primary and secondary antioxidants. Primary antioxidants prevent the formation of free radicals and can be classified into free radical terminators, oxygen scavengers and chelating agents. Radical terminators include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and gallates. The important example of oxygen scavengers includes sulphites, glucose oxidase and ascorbyl palmitate, while chelating agents are heavy metal like iron, copper. Secondary antioxidants like thiodipropionic acid and dilauryl theodipropionate act by breaking down the hydroperoxides formed during lipid oxidation into stable end products. These antioxidants are also used in processed fruits and vegetables, soft drinks, margarine and canned shellfish. There are several antioxidants found in plants that have also prepared been synthetically like gallic acid. The phenolic synthetic antioxidants have similar common biological effect on the molecular, cellular and organ levels. Commonly used synthetic antioxidants like BHA, BHT, ethoxyquin and propyl gallate may produce beneficial interactions such as radioprotection, protection against acute toxicity of chemicals, antimutagenic activity and antitumorigenic action. But beneficial interactions of antioxidants with physical and chemical noxae are contrasted to those leading to adverse effects, for example radiosensitization, increased toxicity of other chemicals, increased mutagen activity and increased tumor yield from chemical carcinogens (*52*, *53*).

Several synthetic chemicals with antioxidant activity also been used in the treatment of different diseases; for example 5-amino-salicylic acid (synthetic antioxidants) was the most effective drug in the treatment of chronic inflammations of the bowel. Recent research has suggested that the antioxidant mexidol may be useful in treatment of acute pancreatitis and it permits to slow down destructive processes in the pancreas, and significantly decrease the number of severe complications of acute pancreatitis and reduce mortality. Intraperitoneal injection of the potent antioxidants carvedilol and melatonin was also found to be beneficial in treatment of chronic fatigue syndrome as it causes significant reduction in immobility. Some statins (example: atorvastatin, simvastatin, pravastatin, rosuvastatin), xanthine oxidase inhibitors like allopurinol also possesses antioxidant activity and used in the treatment of hyperlipoproteinemias, gout respectively. Several synthetic anti-diabetic drugs like glibenclamide, metformin, repaglinide also possess antioxidant activity (*52*–*58*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Antioxidants in Prophylaxis and Therapy

The relation between free radicals, antioxidants and functioning of various organs and organ systems is highly complex and the discovery of redox signaling is a landmark in this regard. In recent years, antioxidants have gained a lot of importance and are emerging as potential as prophylactic and therapeutic agents in many diseases. Free radicals have been associated with pathogenesis of various disorders like cardiovascular diseases, cancer, diabetes, autoimmune diseases, neurodegenerative disorders, gastrointestinal disorders, eye disease, aging and many more. These are the agents, which scavenge ROS and RNS, and prevent the damage caused by them. The discovery of the role of free radicals in pathogenesis of different disease has led to a medical revolution that is assuring a new paradigm of healthcare. Antioxidants may function as immune modulators and can used for prophylaxis or therapy of certain diseases along with the mainstream therapy. Supplements of exogenous antioxidants can act directly to quench the free radical or free radical reactions, prevent lipid peroxidation and also boost the endogenous antioxidant system and hence deliver the prophylactic or therapeutic activity. Many novel approaches and significant findings have come to light in the last few years. The natural food, spices and medicinal plants are rich sources of antioxidants and can be prime source to treat such diseases. Several antioxidants epigallocatechin-3-*O*-gallate, lycopene, ellagic acid, coenzyme Q_{10}, indole-3-carbinol, genistein, quercetin, vitamin C and vitamin E have been found to be pharmacologically active as prophylactic and therapeutic agents. Therefore targeting oxidative stress or boosting the endogenous levels of antioxidants by the use of antioxidants and or is likely to have beneficial outcome in the management of several disorders (*59*, *60*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Antioxidants against Disease

Cardiovascular Disease

Cardiovascular disease is of multifactorial etiology linked with a multiple risk factors like hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity. ROS-induced oxidative stress plays a key role in pathogenesis of different cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure. Oxidative stress is responsible for altering phospholipids and proteins leading to peroxidation and oxidation of thiol groups. Oxidative stress leads the changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins; abnormalities in myocyte function due to increased due to the effects of ROS on subcellular organelles. In general, Ca^{2+} overload can be induced by ROS, which is playing a key role in genesis of myocyte dysfunction (*1*, *4*).

Endothelial dysfunction is one of the major causes of cardiovascular diseases, but it is believed that oxidation of LDL, loss of nitric oxide and vascular inflammatory due to oxidative stress would implicate a potential for antioxidant

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

therapies to ameliorate endothelial dysfunction. The potential effect of both synthetic and natural antioxidant was investigated. Antioxidant vitamins by potentiating endothelial nitric oxide levels and inhibiting vascular inflammation, lipid peroxidation, platelet aggregation, oxidation of LDL can be beneficial to prevent endothelial dysfunction. But results of some results of prospective antioxidant clinical trials have been disappointing. Though *in vitro* study showed the potential effect of α-tocopherol, ascorbic acid and β-carotene, but *in vivo* result is not satisfactory. The biggest doubt, which antioxidants raises is that of suicidal oxidative stress, induced by certain antioxidants, which can act as pro-oxidants in high concentrations and can cause the cell to undergo severe oxidative stress ultimately resulting in suicidal cell death. Antioxidant vitamins like vitamin C, vitamin E and β-carotene produce complicated and conflicting results studied by different researchers. There biggest concern is which antioxidants can act as pro-oxidants and can cause the cell to undergo severe oxidative stress ultimately resulting in suicidal cell death. Further research is required to investigate the *in vivo* effect of certain antioxidant and their mechanism of action. Although cereals, pulses (legume), spices, dark green leafy vegetables such as kale and spinach, citrus fruits, crude palm oil, soybean oil, cod liver oil, sprouts, peppers, whole grain, honey, walnuts and black tea can significantly increase the hepatic antioxidant enzymes reduces the risk of cardiovascular diseases. Some specific fruits, vegetables or legumes can prevent cardiovascular disease induced by oxidative stress, due to presence of unique dietary antioxidant components. Flavanone, flavanol, flavone, isoflavone, phenolic acid, and anthocyanins are natural antioxidant component found in fruit and vegetables. The promising link between the intake of leguminous foods and reduced risk of cardiovascular disease and coronary heart disease has been reported in several researches. These components may reduce oxidative stress by influencing a variety of cellular activities (inhibit lipid oxidation, increase in plasma antioxidant capacity, reduction in platelet aggregation, plasma lipid levels reduction, scavenge free radicals, reduction in intracellular ROS generation, induction of GSH, anti-apoptotic effect, increase in bioavailability of nitric oxide, reduction in matrix metalloproteinase production, anti- inflammatory responses) related to cardiovascular health and decreases the chance of moetality (*61–65*).

Cancer

Free radical induced oxidative stress causes a cellular redox imbalance usually found in various cancer cells compared with normal cells, it may be related to oncogenic stimulation modification of genetic material resulting from “oxidative damage”. DNA mutation is a vital step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly involving such damage in the etiology of cancer. RNS like peroxynitrites and nitrogen oxides, have also been implicated in DNA damage and cancer. ROS causes activation of AP-1 (activator protein) and NF-κB (nuclear factor kappa

B) signal transduction pathways, which in turn lead to the transcription of genes involved in cell growth regulatory pathways and pathogenesis of cancer (*4*, *66*).

Antioxidants may act by preserving normal cell cycle regulation, inhibition of proliferation and inducing apoptosis, inhibition of tumour invasion and angiogenesis, suppression of inflammation, and stimulation of phase II detoxification enzyme activity. Activation of NF-κB can be blocked by several antioxidants, including l-cysteine, *N*-acetyl cysteine (NAC), thiols, green tea polyphenols, and Vitamin E. Antibiotic antioxidants like vitamin C, vitamin E and β-carotene also found to have preventive role against cancer. Photoprotective property of β-carotene is helpful to protect against ultraviolet (UV)-light-induced cancer. Vitamin E plays a key role in immunocompetence (by increasing humoral antibody production, resistance to bacterial infections, cell-mediated immunity, the T lymphocyte response, tumor necrosis factor (TNF) production, and natural killer cell activity), inhibit of mutagen formation, repair of membranes and DNA, and blocking of nitrosamine formation and thus prevent the cancer formation. Vitamin C confers its anti-carcinogenic activity through its effects on blocking the formation of nitrosamines and fecal mutagens, enhance the immune response, and accelerate of detoxification of liver enzymes. Chemotherapy (like bleomycin) and radiation therapy (x-ray and γ-ray) in cancer can generate free radicals and causes threaten the integrity and survival of surrounding normal cells. Antioxidant diet or consumption of antioxidant like vitamins E, C, and selenium may have potential role in enhancing the efficacy of cancer treatment, they may also protect against side effects to normal tissues that are associated with treatment (*4*, *64*, *67*).

Diabetes

Diabetes mellitus is global problem associated with increased formation of free radicals and decrease in antioxidant potential, which results disturbed balance between radical formation and antioxidant protection in normal cell. Both insulin dependent (type 1) and non-insulin-dependent diabetes (type 2) are associated with increased oxidative stress. Hyperglycemia in can also stimulates ROS formation from a variety of sources like oxidative phosphorylation, glucose autooxidation, NAD(P)H oxidase, lipooxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS). Several studies are reported the depletion of antioxidant enzyme levels in patients with diabetes. Oxidative stress has also implicated in the pathogenesis of cardiovascular disease, retinopathy, neuropathy, nephropathy, and erectile dysfunction in diabetes (*4*, *58*).

Antioxidant can confer significant beneficial effect in diabetic patient. Antioxidant vitamins and supplements can help decrease the markers indicative of oxidant stress and lipid peroxidation in diabetic subjects. The intake of dietary antioxidant (total vitamin E, α-tocopherol, γ-tocopherol, β-tocotrienol, and β-cryptoxanthin) was associated with a reduced risk of type 2 diabetes. Phytochemicals with antioxidant activity like cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes also proved beneficial to protect diabetes or protect diabetic

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

complications. Several anti-diabetic and other synthetic drugs with antioxidant activity like angiotensin convertase inhibitors, angiotensin block receptors, melatonin, α-lipoic acid, glibenclamide, allopurinol, metformin, repaglinide, caffeic acid phenethyl ester, carvedilol found beneficial in diabetes and to prevent diabetic complications due to their antioxidant activity (*57*, *58*).

Rheumatoid Arthritis

Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by progressive, erosive, and chronic polyarthritis. The pathogenesis of this disease is linked with the formation of free radicals at the site of inflammation which leads to lipid peroxidation. Oxidative stress causes modification of low density lipoprotein, inactivation of alpha-1-protease inhibitor, DNA damage, lipid peroxidation and heat shock protein ciated with the activation of neutorphil, NADPH oxidase and endothelial cell xanthine dehydrogenase, which contribute significantly to the inflammatory process. Oxidative conditions in synovial tissue are also connected with a higher prevalence of p53 mutations. Decrease concentrations of whole blood glutathione and total thiols were found in patients of rheumatoid arthritis. Decreased in glutathione concentration has been associated with cell damage, depressed immunity and progression of ageing (*4*, *68*, *69*).

Exogenous antioxidants like vitamins and other nutrients appear to be potential agents for therapeutic management in the management of rheumatoid arthritis. Different study reported that use of antioxidants as supplements with the conventional drugs yields even better results as revealed by increase in total thiols, glutathione and vitamin C concentrations and decrease in malondialdehyde concentrations. Some clinical trials have reported that use of antioxidant vitamins as a complementary intervention to help manage the disease. Intake of certain antioxidant micronutrients, particularly β-cryptoxanthin and supplemental zinc, and possibly diets high in fruits and cruciferous vegetables, also confer protective against the development of rheumatoid arthritis. These observations clearly supports the use of antioxidant supplemented drug regimen with conventional drug therapy, and suggests that antioxidants may have an important role to play in this inflammatory disorder, as they lower the oxidative stress and the resultant inflammatory damage. Although, some more clinical trials may be require to evaluate the safety and efficacy of adding on antioxidant therapy for the treatment of rheumatoid arthritis (*68*–*71*).

Neurodegenerative Diseases

Neurodegenerative disorders are a heterogeneous group of diseases of the nervous system, characterized by loss of nerve cells from brain and spinal cord, which leads either functional loss (ataxia) or sensory dysfunction (dementia).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Mitochondrial dysfunctions and excitotoxicity and finally apoptosis have been reported in different neurodegenerative diseases. Free redical induced oxidative stress contribute in several neurodegenerative disorders like such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyolotrophic lateral sclerosis, Huntington's disease, cognitive dysfunction in the elderly, schizophrenia and tardive dyskinesia. Neuronal biochemical composition is mainly vulnerable to ROS and causes peroxidation of unsaturated lipids and oxidative modification. The brain consumes an inordinate fraction (20%) of total oxygen for its relatively small weight (2%) and contains high level of fatty acids which are more susceptible to peroxidation. In addition, brain is lower in antioxidant activity and not enriched in antioxidant defenses compare the other tissue like liver. ROS are highly reactive to different fundamental molecules in cellular pool and initiate cascade of reactions at same time that leads to neuronal cell death, which responsible for neurodegenerative diseases (*72–74*).

Inactivation of oxyradicals by dietary antioxidants like vitamin C, vitamin E, β-carotene can be a important approach in neuroprotection in variety of neurological disorders. Use of antioxidant like α-lipoate, coenzyme Q_{10}, melatonin, phenyl-alpha-tert butyl nitrone, flavanoid, GSH-glycosid, and Euk-8 (a salen-manganese complex) also produced beneficial effect in such diseases. Several clinical evidences suggest that neurodegenerations can be ameliorated upon dietary intake or supplementary intake of natural antioxidants. Several natural antioxidants found to prevent oxidation of proteins, lipid peroxidations and prevent generation of ROS, thus act as upstream therapeutic barrier to oxidative stress. An important futuristic upstream therapeutic approach is to protect death of oxidative stress neuronal death against potential toxic protein formed in neuronal disorders can be the future therapeutic strategy to fight against oxidative stress, and a number of natural and synthetic products have been advised to work in neuroprotection to combat the ill effects free radicals. A promising example include the use of β-amyloid vaccination in Alzheimer's disease that prevents plaque formation and subsequent neuron inflammation, and *Ginkgo biloba*, a Chinese herb has been recognized for its excellent antioxidant properties that restrict β-amyloid toxicity after plaque formation (*72*, *73*, *75*).

Effectively of commercial antioxidants against neurodegenerative diseases depends upon their blood brain barrier (BBB) penetration capacity. However, antioxidants possess widely varying chemical structures and therapeutic use of most of these commercial antioxidants is limited since they do not cross the BBB. Expression of most antioxidant enzymes is controlled by the transcription factor nuclear factor-E2-related factor (Nrf2) and antioxidant response elements (ARE) in the genes encoding enzymatic antioxidants and is induced by oxidative stress. Brain of multiple sclerosis patients showed the enhanced expression of Nrf2/ARE-regulated antioxidants is suggestive of the occurrence of oxidative stress. But treatment with exogenous antioxidant required high quantity, therefore, the induction of endogenous antioxidant enzymes by activators of the Nrf2/ARE pathway and develop a antioxidant which can cross the BBB after systemic administration maybe a sophisticated approach to treat of multiple sclerosis and other such disorder (*24*, *73*, *75*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Gastrointestinal Diseases

Oxidative stress has been evaluated as a causative factor for almost all gastrointestinal diseases and intervention with antioxidants attempted, both dietary as well as synthetic. However, the results in these studies have been diverse and need more epidemiological and clinical research before the findings can be applied unanimously and intervention with antioxidants endorsed. The gastrointestinal tract (GIT) is particularly important part to play in the generation and damage through free radicals and well endowed with the enzymatic machinery necessary to form large amounts of oxygen radicals. Mucosal xanthine oxidase and NADPH oxidase found in the resident phagocytotic leukocytes (macrophages, neutrophils, eosinophils) of the lamina propria are consider as important source of free radicals. Other sources of oxygen radicals in the gastrointestinal tract include ischemia, reperfusion, drug ingestion, diet, and radiation therapy. The transcription factor nuclear factor kB (NFkB) plays a key role in immune responses and inflammation (via cyokines) through regulation of gene expression of cytokines and other immune response genes, which are particularly involved in the pathogenesis of various gastrointestinal disorders; which is redox sensitive and responds to oxidative stress. Recent studies have demonstrated the involvement of oxygen radicals following active episodes of small-intestinal ischemia, ulcerative colitis, gastric ulcer, inflammatory bowel disease, pancreatitis, alcoholic and non-alcoholic liver diseases (*76*, *77*).

Despite the wide range of therapeutic attempts, the management of different gastrointestinal diseases has not yet been solved. Anti-inflammatory, antiulcer, immunomodulatory, cytostatic drugs currently used, have several side effects which can be dangerous also. Therefore, the use of natural and/or synthetic antioxidants might be an additional and adjuvant treatment approach to treat GIT disorders, as it is well established that the direct free radical scavenging effect and/or the membrane protection play an important role in the action mechanism of several old-established drugs. Several clinical and preclinical studies had shown the effective ness of natural or synthetic drugs with antioxidant activity in the management of GIT disorders. Protective effects of natural antioxidants, like vitamins A, C and E have been confirmed in the treatment of gastroduodenal ulcer and gastric cancer. Among the synthetic antioxidants, 5-amino-salicylic acid was found as the most effective drug in the treatment of chronic inflammatory bowel disorder. Several preclinical studies has also reported the effectiveness of several phyto-constituent in the treatment of GIT disorders and which is due to their antioxidant potential. A study in 123 alcoholic cirrhoses patient showed that supplement with sadenosyl methionine, a key methyl donor in GSH synthesis improved survival and delayed the need for liver transplantation. Several natural antioxidants found beneficial in the treatment of non-alcoholic fatty liver diseases, viral liver diseases, and acute liver failure. Several investigations reported increased oxidative stress and low level of antioxidant in patient with chronic pancreatitis. The clinical trial also reported the significant improvement in the quality of life and reduction in pain due to combined antioxidant supplementation in patients with chronic pancreatitis. Therefore it is believed that in such conditions, supplementation with antioxidants may break the vicious cycle and help curb the progression of disease. Though, a recent randomised controlled trial in alcoholic liver diseases patients with antioxidant (*N*-acetyl cysteine, vitamins A and E, biotin, selenium, zinc, manganese, copper, magnesium, folic acid and coenzyme Q) and clinical studies with antioxidant supplement in acute pancreatitis have hitherto been unable to establish the efficacy of therapy. Therefore large clinical trials of antioxidant supplementation are essential to explore the extent of antioxidant treatment benefit and establish the hypothesis regarding antioxidant supplementation in various diseases. But it is important to develop new and effective synthetic antioxidants, and widely employ them therapeutically to treat such diseases (*54*, *76*, *77*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Renal Disorders

Renal system is also susceptible to ROS induced damage, and connection between oxidative stress and renal disease are well established. Vascular (endothelial and smooth muscle cells), glomerular (endothelial and mesangial) and tubular (proximal, distal and collector) cells of renal structure are capable to produce ROS due to stimulating factor like drugs, acute hypertension, radiation exposure and hyperoxia. In addition, large amounts of ROS are also generating by granulocytes, monocyte-macrophages and platelets, which are present in many inflammatory renal process (vasculitis, glomerulonephritis, pyelonephritis). Chronic renal failure oxidative stress can be considered as a potentially major cause of patient morbidity and mortality. It may also be implicated in the pathogenesis of atherosclerosis, diabetes, malnutrition, anaemia, dialysis-induced amyloidosis, and perhaps increased risk of cancerogenesis in these patients (*78*, *80*).

Ascorbic acid is most prominent antioxidants, exerting beneficial effects by an inhibition of lipid peroxidation and by reducing endothelial dysfunction. Although deficiency of vitamin C can be observed in chronic renal failure patients, but its administration in these patients requires deliberation; because excessive intake of vitamin C in food or as supplementation may lead to its excessive serum levels, resulting in hyperoxalaemia that may contribute to vascular disease in uraemic patients. Selenium is a cofactor of GPx and deficiency of selenium responsible for reduction in the activity of this enzyme resulting in increased oxidative stress induced diseases like proteinuria and glomerular esclerosis. Selenium and vitamin E supplementation found effective in proteinuria in rats. Vitamin E supplement also probed beneficial in to slow the rate of decline in kidney function in chronic renal failure (*78–80*).

Pulmonary Disorders

The pathogenesis of chronic pulmonary disorders like asthma and chronic obstructive pulmonary disease (COPD) is complex, which involves both airway inflammations with an oxidant/antioxidant imbalance. An air pollutant such as ozone and cigarette smoking, infections, and other allergens increases generation

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

of free radicals and amplify the risk of pulmonary disorders. ROS have been shown to be associated with the pathogenesis of asthma and lung injury by causing direct oxidative damage to epithelial cells and cell shedding. ROS also evoke bronchial hyper-reactivity as well as directly stimulating histamine release from mast cells and mucus secretion from airway epithelial cells (*81*, *82*).

In a separate study reported the effectiveness of antioxidant therapy in the treatment of asthma and COPD. Administration of vitamin E, vitamin C, combination of vitamin E and C, selenium, zinc, and garlic oil, in addition to their classical treatment of asthma showed beneficial effect and reduce the number of daily attacks. Another case-control study showed that symptomatic asthma in adults is associated with a low dietary intake of fruit, the antioxidant nutrients vitamin C and manganese, and low plasma vitamin C levels. Intake of dietary antioxidant also found beneficial in management of asthma. Several thiol antioxidants and mucolytic agents, such as glutathione, *N*-acetyl-L-cysteine, *N*-acystelyn, erdosteine, fudosteine and carbocysteine; Nrf2 activators; and dietary polyphenols (curcumin, resveratrol, and green tea catechins/quercetin) have been reported to increase intracellular thiol status along with induction of GSH biosynthesis and might be useful in the treatment of COPD. Vitamin E and C can protect cellular components from many types of oxidative stress by both free radical scavenging mechanism and stabilizing the cell membranes. Vitamin E is a lipophilic chain-breaking antioxidant that acts by preventing the chain reaction involved in lipid peroxidation, while vitamin C is more hydrophilic and acts to quench radicals inside the cell. Flavanoids also reduce asthma inflammation through antioxidant, antiallergic, and antiinflammatory properties. They are avid scavengers of nitric oxide, can decrease histamine release, arachidonic acid metabolism, and cytokine productions. Increase in selenium intake may suppress asthma inflammation by saturating glutathione peroxidase and down-regulates the transcription factor NF-κB. Therefore the use of antioxidants and targeting oxidative stress or boosting the endogenous levels of antioxidants is likely to have beneficial outcome in the treatment of pulmonary disorders (*81–84*).

Eye Disorders

Eye is the most susceptible organ to oxidative damage caused by light, toxins (smoke), atmospheric oxygen, and abrasion. Oxidative stress recognised as important cause of several eye disorder like cataracts, glaucoma, and macular degeneration. Free radical theory of ageing is related with the aetiology of eye diseases, which postulates that ageing and age-related diseases result from the accumulation of cellular damage from ROS. Ultraviolet increases generation of ROS, the conversion of this light energy into a nerve impulse by the photoreceptors generates more free radicals such as hydrogen peroxide, superoxide and hydroxyl radicals. Proteins in the lens are unusually long lived and are subjected to extensive oxidative damage. The decrease in the Na+/K+ATPase results the inability to maintain steady concentrations of Na^+, K^+, and Ca^{++} within the lens, which is thought to be associated with oxidative damage to the sulfhydryl portions of the molecule, usually protected by the interaction of several antioxidants (*85–87*).

Antioxidants are found to have beneficial in the management of eye disorders. Vitamin E is found in human lenses and experimental studies have demonstrated that vitamin E is able to reverse cataract formation. Retinol has also found in human lenses but results from epidemiological studies are controversial. Carotenoids are also thought to play a role in management of cataract. Different carotenoids like beta-carotene, alpha-carotene, betacryptoxanthin, lycopene, zeaxanthin and lutein may confer beneficial effect in such conditions. Population-based cohort study, higher dietary lutein and zeaxanthin intake reduced the risk of long-term incident age related macular degenerations. Animal experiments have shown evidence for a protective role of selenium on cataract formation. Many more clinical trials have needed to be conduct to find the protective effect of other promising phytochemicals with antioxidant and anti-inflammatory properties including flavonoids (catechins, Ginkgo biloba, bilberry, grape seed extract, green tea), anthocyanins (bilberry, blueberry, black currant), and stilbenes (resveratrol) etc (*85–89*).

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Infertility and Pregnancy

ROS can have beneficial or detrimental effects on reproductive system depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS. Free radicals can act as key signal molecules modulating various reproductive functions and can influence the oocytes, sperm, and embryos in their microenvironments, for example follicular fluid, hydrosalpingeal fluid, and peritoneal fluid. These microenvironments have a direct influence on superiority of oocytes, sperm oocyte interaction, implantation, and early embryo development. Oxidative stress has been implicated in male and female infertility (*90*). ROS are known to be generated from spermatozoa and leucocytes, but elevated production of ROS in semen affects sperm function, especially fusion events associated with fertilization, and direct infertility in male. Increased ROS levels also correlate negatively with sperm concentration and sperm motility. Spermatozoa are highly susceptible to the damage induced by excessive ROS because the plasma membrane of spermatozoa contain large quantities of polyunsaturated fatty acids (PUFA) and cytoplasm contains low concentrations of scavenging enzymes. Peroxidative damage causes caused by free radicals results impaired sperm function (*90–92*).

Oxidative stress influences multiple physiological processes, from oocyte maturation to fertilization, embryo development and pregnancy. Oxidative stress influences both implantation and early embryo development which decides a successful pregnancy. Free radical generation. The role of oxidative stress has been demonstrated in female infertility, including fetal dysmorphogenesis, abortions, and intrauterine growth restriction. Oxidative stress has been implicated in many of the causes of infertility, such as endometriosis, polycystic ovarian disease, unexplained infertility, tubal infertility, and recurrent pregnancy loss. Oxidative stress may influence the expression of cytokine receptors in the placenta, cytotrophoblasts, vascular endothelial cells, and smooth muscle cells. Increase in ROS is also involved in defective embryo development and retardation of embryo growth, which is attributed to induced cell-membrane damage, DNA damage, and apoptosis (*92*, *93*).

Treatment with antioxidant found to have beneficial effect in both male, female and to induce normal pregnancy. Antioxidant supplements play a vital role and help endogenous enzymatic antioxidants to act nullify the oxidative stress. Different research showed that endogenous and exogenous antioxidants such as SOD, CAT, glutathione, vitamin E, vitamin C, *N*-acetyl cysteine, albumin, taurine and hypotaurine prevents sperm mortality and *N*-acetyl cysteine and coenzyme Q_{10} increases sperm motility by reducing oxidative stress. For example, (a) oral supplementation coenzyme Q_{10} was shown to improve fertilization rate, (b) administration of vitamin C along with vitamin E and glutathione showed significant reduction in hydroxyl glutathione levels in spermatozoa and also causes increase in sperm count, (c) vitamin E supplement to a group of asthenozoospermic patients with normal female partners showed a significant decrease in lipid peroxidation and increased motility and pregnancy rates. Another study on human suggests that higher antioxidant consumption is associated with increased sperm numbers and motility, and to some extent it may attenuate the impact of age on sperm motility in healthy, non-smoking volunteer (*91*, *94*).

Various antioxidant systems, like SOD, CAT, vitamin E, glutathione and carotenoids play a vital role to control ROS at physiological levels within the ovary. So that theca interna cells can protect the oocyte from excess ROS during its maturation. Trasferrin also suppress ROS generation and important antioxidant factor important for healthy follicle development. Vitamin C also confers a protective effect within the follicle. Pathophysiology of polycystic ovarian disease and endometriosis associated infertility clearly has shown the association with oxidative stress, and drugs such as Rosiglitazone maybe effective by decreasing the levels of oxidative stress. *N*-acetyl-cysteine, acknowledged replenishing stores of the antioxidant glutathione, on insulin secretion and peripheral insulin resistance in subjects with polycystic ovarian syndrome. Recently RU486, pentoxifylline, curcumin, caffeic acid phenethyl ester have been shown to be effective in endometriosis, and their antioxidant activity may contribute in this regard. Moreover vitamin C and E supplementation appears to be effective in preventing or reducing disease severity associated with preeclampsia. Demand for enzymatic antioxidant defense is increased in embryos and oocytes and their tubal and follicular fluid microenvironments during pregnancy, and several studies also reported the linkage of significant decrease in antioxidant level (like SOD, Vitamin E, β-carotine, etc.) is associated with miscarriage and abortion. Vitamin C and E are may play a key role in compensating for the oxidative burst during early pregnancy and reduce the risk of pregnancy loss. Several studies reported that folic acid supplements had better quality oocytes and a higher degree of mature oocytes, and may reduce risk of having a baby with a neural-tube defect. Although another study has failed to prove that folic acid supplements can decrease the risk of miscarriage. Report also showed that of omega-3 supplements have been used in prevention of recurrent miscarriage with antiphospholipid syndrome, and melatonin may be essential for successful

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

pregnancy and prevention of spontaneous abortion. Therefore several further investigations on human are needed to confirm their action (*92*, *93*, *95*).

Aging

Ageing is inevitable process may be defined as a progressive decline in the physiological functions of an organism after the reproductive phase of life. Although the rate of aging is nutrition and oxidative stress dependent, oxidative stress is responsible for damage of different cell components and believed to be one of the key components of age related disorders. Two main theories regarding the process of ageing have been proposed: genetic theory and damage-accumulation theories which involve free radical theory, glycation theory, error catastrophe theory, membrane theory, entropy theory and others. Among this "free radical theory" is probably the most complex approach and based on the fact that the random deleterious effects of free radicals produced during aerobic metabolism cause damage to DNA, lipids, and proteins and accumulate over time (*4*, *96*).

The human body has several endogenous antioxidants to eliminate free radicals. But relative depletion of those enzymes causes oxidative stress. Decreases in free radicals are generation will decrease the chances of oxidative stress induced injury to the mitochondria and their DNA, which overall should reduce aging and age- associated diseases. Since free radical damage to biological molecules has been implicated as the principal cause of aging, therefore attempts to attenuate free radical reactions through exogenous antioxidant. Diet restriction or caloric-restricted diets showed the effectiveness to increase life span in mice and consider as an approach that can increased the expression of antioxidant enzymes such as SOD, CAT. Exercise is approach training raised the levels of antioxidant enzymes in both skeletal and cardiac muscle. Many antioxidant compounds have been shown to increase the average or mean life span of an organism/experimental animals like *N*-acetylcysteine, a synthetic antioxidant and butyl hydroxytoluene. It is also reported that vitamin C did have some life-extending effects, and vitamin E produced a marginal increase on median life span with no influence in maximum life span. Treatment with some antioxidants, such as ascorbic acid, tocopherols, and polyphenols, carotenoids, coenzyme Q_{10}, ergothioneine may be effective to enhance resistance to oxidative stress and prevent/improve skin aging (*96–98*).

Free radicals also found to be associated with several other disorders and the use of antioxidant probably may play an important role. Disorders in premature infants like bronchopulmonary, dysplasia, periventricular leukomalacia, intraventricular hemorrhage, retinopathy of prematurity and necrotizing enterocolitis are shown to be associated with oxidative stress and therefore it may be quite interesting that the use of antioxidant in management of such disorders. Several antioxidants are also possessing antimicrobial activity, therefore food industries are regularly using antioxidants which may contribute to controlling the growth of some food-borne pathogens. Antioxidant property of a substance also may produce beneficial effect to accelerate wound healing. The discussion

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

clearly shows that antioxidant property of a substance or intake of antioxidant substance is may be the future treatment strategy to treat or prevent the health problems. Though some of the antioxidant constituents has been proved clinically effective but lot more research and clinical trial still essential to explore any adverse effect or interactions by other antioxidant constituents (*2*, *99*). Role of different antioxidants and their sources are tabulated in Table III.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table III. Different antioxidants and their effects

Antioxidants	*Location/Sources*	*Remarks*
Superoxide dismutase (SOD)	Cytosol, mitocondria, nucleus, plasma	Dismutation of superoxide to hydrogen peroxide (H_2O_2)
Catalase (CAT)	Peroxisomes	Dismutation of H_2O_2 to molecular oxygen and water
Glutathione peroxidase (GPx)	Cytosol, mitochondria	Reduction of H_2O_2 and other hydroperoxides, lipid peroxides, lipoxygenase products
Glutathione reductase (GR)	Cytosol, mitochondria	Reduction of low molecular weight disulfides
Glutathione (GSH)	A tripeptide is present in high concentrations in most eukyrotic cells. It present within the cytosol of cells and is the major intracellular nonprotein thiol compound	Substrate in GSH redox cycle, act as a reductant, reducing H_2O_2 directly to water with the formation of GSSG. It also reacts with superoxide anion, hydroxyl, and alkoxyl radical directly by a radical transfer process and inhibits tissue damage. GSH is capable of scavenging ROS directly or enzymatically via GPx.
Uric acid	Wide distribution	Uric acid is a powerful antioxidant and is a scavenger of singlet oxygen and radicals like superoxide anion, hydroxyl, and alkoxyl radical and binds transition metals.
Cysteine	Wide distribution	Cysteine is also a vital component for the synthesis of glutathione and can reduce organic compounds by donating e- from SH groups. N-acetyl-L-cysteine (NAC) is a derivative of cysteine act as glutathione precursor and Scavenges of H_2O_2 and peroxide
CoQ_{10}	synthesized in human cells and also found in wheat bran, fish, organ meats	It inhibit lipid peroxidation, reduces mitochondrial oxidative stress, and also able to recycle vitamin E.

Continued on next page.

Table III. (Continued). Different antioxidants and their effects

Antioxidants	*Location/Sources*	*Remarks*
Transferrin	It is a major iron transporting protein in the body	It bind free iron salts, which can leads to the generation of reactive oxygen species
Lactoferin	It is a milk protein found extracellularlly	Similar action like transferring to helps in iron binding
Ceruloplasmin	A metal binging protein in extracellularlly	It is a copper binding protein. It catalyses the oxidation of Fe^{2+} to Fe^{3+} while oxygen is reduced to water.
Bilirubin	Blood stream tissue, plasma and extravascular place. It is Principal component of RBC.	It is an end product of heme catabolism, generally viewed as cytotoxic, lipid-soluble waste product. But at micromolar concentrations in vitro, efficiently scavenges peroxyl radicals and protects albumin-bound linoleic acid from peroxyl radical-induced oxidation.
Vitamin E	Present in relatively high concentrations in both cells and mitochondrial membranes. Found in amla (Indian gooseberry), lemon, oranges, groundnut oil, olive oil, palm oil, cashew nuts, germinated pulses, rasins	Direct scavenging of superoxide, hydroxyl radicals, upregulation of antioxidant enzymes, breaks lipid peroxidation chain reactions.
Vitamin C	ICF, ECF, and also found in lemon, oranges, olive oil, palm oil, cashew nuts, germinated pulses,	Scavenges superoxide, hydroxyl radicals, neutralize oxidants from stimulated neutrophils, regenerates vitamin E
Carotenoids: α-carotene, β-carotene, γ-carotene, crocin, β-cryptoxanthin, lycopene, lutein, zeaxanthin, bixin, astaxanthin, capsorubin, canthaxanthin	apples, banana, berries, grapes, jackfruit, kiwi, lemon, mango, pineapple, orange, papaya, watermelon, sweet potato, plum, carrot, asparagus, beet beetroot, brinjal, broccoli, brussels sprouts, tomatoes, spinach, cauliflower, corn, onions, cabbage, beans, pumpkin, cucumber, mushroom,	Antioxidant activity of carotenoids is based on their singlet oxygen quenching activity. Carotenoids are found useful in cancer, cardiovascular diseases, cataract, HIV infection. Carotein useful to decrease the risk of cardiovascular diseases and certain cancers; lutein and zeaxanthin effective in eye disorders; α-and β-carotein and β-cryptoxanthin can able to be converted to Vitamin A and its related role in the development an d disease prevention; lycopene is effective

Continued on next page.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Table III. (Continued). Different antioxidants and their effects

Antioxidants	*Location/Sources*	*Remarks*
	chillies, red palm oil, Milk, yogurt, eggs and medicinal plants	in cancer, cardiovascular diseases, osteoporosis, emphysema.
Flavonoids		
Anthocyanidins (cyanidine, delphinidine, petunidine),	Blackberries, blueberries, strawberries, plum	Flavanoids have capable to scavenge hydroxyl radical, superoxide anions and lipid peroxy radicals. Different flavonoid have found effective in several clinical and preclinical studies. Some of the important effect of favonoids includes antiulcer, antiviral, anti inflammatory, anti arthritis, anti osteoporotic, antidiarrheal, antiatherosclerosis anti platelet aggregation and anti thrombogenic effects,
Flavonols (isorhamnetin, kaempferol, quercetin, myricetin)	Apple, tomato, cherry, broccoli, onion	
Isoflavonoids (anisole, cumestrol, daidzein, genistein)	Soyabeen, soy foods, legumes (greengram, lupin peas, soy beans, white and horse gram)	
Flavones (apigenin, luteolin)	Green leafy spices (parsley)	
Flavanones (naringenin, hesperetin. eridictyol)	Citrus fruits (orange, lemon)	
Flavanol (epicatechin, catechin, glycocetachine),	Tea, red grapes, chocolate, cocoa beverages, red wine	
Other polyphenols: cinnamic acid, coumarin, condensed tannins, hydroxybenzoic acid (gallic aid, protocatechuic acid, vanillic acid), hydroxycinnamic acid (caffeic acid, caftaric acid, chlorogenic acid, coumaric acid, ferulic acid, sinapic acid), proanthocyanidins,	Green leafy vegetables, plum, blackberries, blueberries, strawberries, apple, tomato, cherry, broccoli, onion	

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Risks of Antioxidants

Our endogenous antioxidant defense systems are incomplete without exogenous antioxidants such as vitamin C, vitamin E, carotenoids and polyphenols are playing an essential role in maintaining redox balance. However, isolated compounds at high concentration may be toxic, owing to pro-oxidative effects or their potential to react with beneficial concentrations of ROS normally present at physiological conditions that are required for optimal cellular functioning. Natural and dietary antioxidants have been found to useful in preventing or treatment in certain diseases. However, though it is apparent that natural antioxidants have many positive effects on health, but they could also have harmful effects if taken in excess. If natural antioxidants are taken in greater than certain concentrations, they can create significant, sometimes even deadly, physiological effects. Generally it was estimated that vitamin C, vitamin E and carotenoids range between high micromolar and low millimolar levels in human plasma and organs, while polyphenol concentrations found in the high nanomolar to low micromolar range. An interactive and often synergistic action between endogenous and exogenous antioxidants helps to maintain a balance between oxidation and antioxidation. Consumption of vitamin C more than 3000 mg/day for several days can lead to illnesses such as kidney stones, and an increased need for oxygen. Also, intake of vitamin C with any acid can lead to excess uric acid excretion and erosion of dental enamel. Few studies also reported that vitamin E supplements may amplify the risk of bleeding in certain individuals. Vitamin A overdoses (1600-3200 mg/kg) for extended period can also lead to symptoms such as fatigue, breast soreness, gastrointestinal stress, renal problems, vascular inflammation, and thyroid problems. Coenzyme Q10 also may cause severe haemorrhage if taken in very large quantities, and can act like pro-oxidant in high concentrations. Some reports also suggested that β-carotene supplementation can produce significant increase in stroke incidence and overall cardiovascular deaths. Some of the studies reported that β-carotene supplements may increase the risk of lung cancer in smokers. The dietary antioxidants like β-carotene, vitamin C, vitamin E and other produce complicated and conflicting results in cardiac health studied by different researchers. The intake of active antioxidant compounds will help to prevent complex multi-factorial diseases and may slow their development or inhibit their progression. Antioxidant foods generally are considered safe, but several antioxidant supplements are reported to have adverse effects. Therefore, the use of these compounds and consumption of these as complex foods within the normal diet or as individual or mixed supplements, and the suitable doses that confer health benefits without additional risk, have yet to be identified. Synthetic antioxidants at very large doses also may produce toxic effects. For example BHT and BHA found to produce alterations in enzyme activity, lipid concentration and carcinogenic effects. It is believed that degradation products of those chemicals mainly responsible for such toxic effects. Even though at normal level synthetic antioxidants seem to pose no side effect, but tong term use may led to chronic health problems (*25*, *52*, *100*, *101*). Therefore public awareness and proper investigation regarding this is the need to minimize the toxic effect of antioxidants.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Perspectives and Future Directions

Despite of the existence of endogenous defense mechanisms against ROS, it has been suggested that we are more susceptible to ROS induced oxidative stress due to change in lifestyle, food habits, pollution, and stress. More than 100 disorders have been reported as the ROS-mediated disorders. The association of ROS in aging has been well documented. Modern science and research have begun to unravel the molecular components of free-radical biology and biological inter-relationships of these components in mediating various disease processes for a better understanding and exploitation in biomedical/clinical sciences. Several clinical studies and basic research have shown that depletion of endogenous enzyme in certain pathological may be mitigated by the use of exogenous antioxidants might. Sustained interest in the use of antioxidants for management of human disease and the role of dietary antioxidant in prevention of disease offer better understanding for the development of newer and better therapeutic entities with antioxidant activity. Parallel identification, development of natural or synthetic antioxidants and their suitable formulation can provide enormous scope for better treatment of several diseases. On-going studies continue to produce epidemiological evidence suggesting that antioxidant rich foods or antioxidant supplements reduce the risk of chronic disease and promote wellness. Several factors like poor solubility, inefficient permeability, instability due to storage of food, first pass effect, and GI degradation need to be improved antioxidants are to be developed as drug targets. Therefore modification in dosage form, physicochemical characteristics, biopharmaceutical properties and pharmacokinetic parameters are important to consider in the drug development process. Progression in both the therapeutic and nutritional fields with antioxidants has not only been punctuated by some successes, but by various spectacular failures as well *((21*, *59*, *101–104*).

Today, encouraging a varied diet (healthy food with antioxidant activity) remains the best advice in garnering the benefits of antioxidants and the many other bioactive components available from food. Concurrently, it is also necessary to avoid oxidant sources (cigarette, alcohol, exposure of chemicals, stress etc) to remain healthy. Continued research is needed to identify the therapeutically important constituents and dietary components that have antioxidant action, quantify these components and assess their potential for *in vivo* antioxidant activity and their interactions with target tissue. It is also imperative to reveal the relationship between intake of antioxidants and their dose-dependent functional effects. In the light of present understanding the appropriate role for antioxidants in human health will become more apparent. As clinical evidence emerges and our understanding of genomic differences improves, the specific role of antioxidants with variation of species or genetic differences need to be identify. The future holds great promise for discoveries of new knowledge about free radical biology and antioxidants, and for turning this basic knowledge into practical use for ensuring a healthy life. Developing coordinated research collaborations involving biomedical scientists, phytochemical researchers, nutritionists and physicians is a critical first step evaluating the role of antioxidants in health and disease for the coming decades.

References

1. Pham-Huy, L. A.; He, H.; Pham-Huy, C. *Int. J. Biomed. Sci.* **2008**, *4*, 89–96.
2. Sen, S.; Chakraborty, R.; Sridhar, C.; Reddy, Y. S. R.; De, B. *Int. J. Pharma. Sci. Rev. Res.* **2010**, *3*, 91–100.
3. Devasagayam, T. P. A.; Tilak, J. C.; Boloor, K. K.; Sane, K. S.; Ghaskadbi, S. S.; Lele, R. D. *J. Assoc. Physicians. India* **2004**, *52*, 794–804.
4. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.
5. Gerschman, R.; Gilbert, D. L.; Nye, S. W.; Dwyer, P.; Fenn, W. O. *Science (Washington, D.C.)* **1954**, *119*, 623–626.
6. Gupta, V. K.; Sharma, S. K. *Natural Product Radiance* **2006**, *5*, 326–334.
7. Percival, M. *Clinical Nutrition Insights*. 1998, (NUT031 1/96 Rev. 10/98). http://www.acudoc.com/Antioxidants.PDF (accessed April 2, 2011).
8. Rathore, G. S.; Suthar, M.; Pareek, A.; Gupta, R. N. *J. Nat. Pharmaceuticals* **2011**, *2*, 2–14.
9. Fang, Y.; Yang, S.; Wu, G. *Nutrition* **2002**, *18*, 872–879.
10. Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 3rd ed.; Oxford University Press: New York, USA, 1999; pp 10-121.
11. Sarma, A. D.; Mallick, A. R.; Ghosh, A. K. *Int. J. Pharma Sci. Res.* **2010**, *1*, 185–192.
12. Bagchi, K.; Puri, S. *East. Mediterr. Health J.* **1998**, *4*, 350–360.
13. Lander, H. M. *FASEB J.* **1997**, *11*, 118.
14. Jacob, R. A. *Nutr. Res.* **1995**, *15*, 755–766.
15. Sies, H. *Oxidative Stress: Oxidants and Antioxidants*; Academic Press: London, UK, 1991.
16. Demir, S.; Yilmaz, M.; Koseoglu, M.; Akalin, N.; Aslan, D.; Aydin, A. *Turk. J. Gastroenterol.* **2003**, *14*, 39–43.
17. Langseth, L. *Oxidants, Antioxidants and Disease Prevention*; International Life Science Institute: Belgium, 1996.
18. Devasagayam, T. P. A.; Boloor, K. K.; Ramsarma, T. *Indian J. Bioche. Biophys.* **2003**, *40*, 300–308.
19. Kuraoka, I.; Robins, P.; Masutani, C.; Hanaoka, F.; Gasparutto, D.; Cadet, J.; Wood, R. D.; Lindah, T. *J. Biol. Chem.* **2001**, *276*, 49283–49288.
20. Serafini, M. *Medicine* **2006**, *34*, 533–535.
21. Benzie, I. F. F. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2003**, *136*, 113–126.
22. Carlsen, M. H.; Halvorsen, B. L.; Holte, K.; Bohn, S. K.; Dragland, S.; Sampson, L.; Willey, C.; Senoo, H.; Umezono, Y.; Sanada, C.; Barikmo, I.; Berhe, N.; Willett, W. C.; Phillips, K. M.; Jacobs, D. R., Jr; Blomhoff, R. *Nutr. J.* **2010**, *9*, 1–11.
23. *Antioxidant Supplements for Health: An Introduction 2011*; U.S. Department of Health and Human Services, U.S. Government Printing Office: Washington, DC, 2000.
24. *Human vitamin and mineral requirements 2001;* Report of a joint FAO/WHO expert consultation Bangkok, Thailand, Food and Nutrition Division FAO: Rome, 2001.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

25. Bouayed, J.; Bohn, T. *Oxid. Med. Cell Longev.* **2010**, *3*, 228–237.
26. Nunez, M. J.; Costoya, N. *Electronic Journal of Environmental, Agricultural and Food Chemistry* **2008**, *7*, 3335–3342.
27. Wohlmuth, H. In *An Introduction to Complementary Medicine*; Robson, T., Ed.; Allen & Unwin: Victoria, Australia, 2004; pp 191-212.
28. Salim, A. A.; Chin, Y. W.; Kinghorn, A. D. In *Bioactive Molecules and medicinal Plants*; Ramawat, K. G.; Merillon, J. M., Ed.; Springer: Berlin, Germany, 2008; pp 1-18.
29. Kamboj, V. P. *Curr. Sci.* **2000**, *78*, 35–39.
30. Atawodi, S. E. *Afr. J. Biotechnol.* **2005**, *4*, 128–133.
31. Adewusi, E. A.; Moodley, N.; Steenkamp, V. *S. Afr. J. Bot.* **2011**in press.
32. Chen, I.; Chan, H.; Yang, H.; Chen, G. *Journal of Food and Drug Analysis* **2004**, *12*, 29–33.
33. Gan, R.; Xu, X; Song, F.; Kuang, L.; Li, H. B. *J. Med. Plant. Res.* **2010**, *4*, 2438–2444.
34. Li, C; Liu, S. *Modern Pharmaceutical Research* **2009**, *2*, 31–35.
35. Chon, S.; Heo, B.; Park, Y.; Cho, J.; Gorinstein, S. *J. Sci. Food Agric.* **2008**, *88*, 1963–1968.
36. Cho, E. J.; Yokozawa, T.; Rhyu, D. Y.; Kim, S. C.; Shibahara, N.; Park, J. C. *Phytomedicine* **2003**, *10*, 544–551.
37. Kim, S. I.; Sim, K. H.; Choi, H. *Mol. Cell Toxicol.* **2010**, *6*, 279–285.
38. Jung, H. A.; Kim, A. R.; Chung, H. Y.; Choi, J. S. *Arch. Pharm. Res.* **2002**, *25*, 865–872.
39. Nanasombat, S.; Teckchuen, N. *J. Med. Plant. Res.* **2009**, *3*, 443–449.
40. Makchuchit, S.; Itharat, A.; Tewtrakul, S. *J. Med. Assoc. Thai.* **2010**, *93* (Suppl. 7), S227–S235.
41. Al-Mustafa, A. H.; Al-Thunibat, O. Y. *Pak. J. Biol. Sci.* **2008**, *11*, 351–358.
42. Al-Ismail, K.; Hamdan, M.; Al-Delaimy, K. *Jordan J. Agric. Sci.* **2006**, *2*, 64–74.
43. Salazar-Aranda, R.; Perez-Lopez, L. A.; Lopez-Arroyo, J.; Alanis-Garza, B. A.; de Torres, N. W. *J. Evid. Based Complementary Altern. Med.* **2011**, *2011*, 1–6.
44. Ruiz-Teran, F.; Medrano-Martinez, A.; Navarro-Ocana, A. *Afr. J. Biotechnol.* **2008**, *7*, 1886–1893.
45. Ramos, A.; Visozo, A.; Piloto, J.; Garcia, A.; Rodriguez, C. A.; Rivero, R. *J. Ethnopharmacol.* **2003**, *87*, 241–246.
46. Huerta, V.; Mihalik, K.; Maitin, V.; Crixell, S. H.; Vattem, D. A. *J. Med. Plant. Res.* **2007**, *1*, 38–49.
47. Velazquez, E.; Tournier, H. A.; Mordujovich, B. P.; Saavedra, G.; Schinella, G. R. *Fitoterapia* **2003**, *74*, 91–97.
48. Lock, O.; Castillo, P.; Doroteo, V.; Rojas, R. *III WOCMAP Congress on Medicinal and Aromatic Plants*. 2005, *1*. http://www.actahort.org/books/675/675_13.htm, (accessed Apr 10, 2011).
49. Antal, D. S. *Analele Universitatii din Oradea – Fascicula Biologie* **2010**, *17*, 14–22.
50. Kratchanova, M.; Denev, P.; Ciz, M.; Lojek, A.; Mihailov, A. *Acta Biochim. Pol.* **2010**, *57*, 229–234.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

51. Krishnaiah, D.; Sarbatly, R.; Bono, A. *Biotechnol. Mol. Biol. Rev.* **2007**, *1*, 97–104.
52. Venkatesh, R.; Sood, D. *Review of the Physiological Implications of Antioxidants in Food*. Bachelor of Science Interactive Qualifying Project. Worcester Polytechnic Institute, January 2011.
53. Kahl, R. *Toxicol.* **1984**, *33*, 185–228.
54. Feher, J.; Pronai, L. *Orv Hetil.* **1993**, *134*, 693–696.
55. Singh, A.; Naidu, P. S.; Gupta, S.; Kulkarni, S. K. *J. Med. Food* **2002**, *5*, 211–220.
56. Kuznetsov, N. A.; Rodoman, G. V.; Brontvein, A. T.; Laberko, L. A.; Malgina, N. V.; Sumedi, I. R. *Khirurgiia (Mosk)* **2005**, *3*, 36–39.
57. Montonen, J.; Jarvinen, R.; Knekt, P.; Reunanen, A. *Diabetes Care* **2004**, *27*, 362–366.
58. Rahimi, R.; Nikfar, S.; Larijani, B.; Abdollahi, M. *Biomed. Pharmacother.* **2005**, *59*, 365–373.
59. Ratnam, D. V.; Ankola, D. D.; Bhardwaj, V.; Sahana, D. K.; Kumar, M. N. V. R. *J. Control. Release* **2006**, *113*, 189–207.
60. Aher, V. D.; Wahi, A.; Pawdey, A. M.; Sonawane, A. *Int. J. Curr. Pharma. Res.* **2011**, *3*, 8–10.
61. Blomhoff, R. *Curr. Opin. Lipidol.* **2005**, *16*, 47–54.
62. Maxwell, S. R. J.; Lip, G. Y. H. *Br. J. Clin. Pharmacol.* **1997**, *44*, 307–317.
63. Tandon, V. R.; Verma, S.; Singh, J. B.; Mahajan, A. *Drug Rev* **2005**, *7*, 61–64.
64. Hercberg, S.; Galan, P.; Preziosi, P.; Alfarez, M.; Vazquez, C. *Nutrition* **1998**, *14*, 513–520.
65. Wang, S.; Melnyk, J. P.; Tsao, R.; Marcone, M. F. *Food Res. Int.* **2011**, *44*, 14–22.
66. Valko, M.; Rhodes, C. J.; Moncola, J.; Izakovic, M.; Mazur, M. *Chem. Biol. Interact.* **2006**, *160*, 1–40.
67. Borek, C. *Integr. Cancer Ther.* **2004**, *3*, 333–341.
68. Jaswala, S.; Mehta, H. C.; Sood, A. K.; Kaur, J. *Clin. Chim. Acta* **2003**, *338*, 123–129.
69. Mahajan, A.; Tandon, V. R. J. *Indian Rheumatol. Assoc.* **2004**, *12*, 139–142.
70. Cerhan, J. R.; Saag, K. G.; Merlino, L. A.; Mikuls, T. R.; Criswell, L. A. *Am. J. Epidemiol.* **2003**, *157*, 345–354.
71. Vugt, R. M. V.; Rijken, P. J.; Rietveld, A. G.; Vugt, A. C. V.; Dijkmans, B. A. C. *Clin. Rheumatol.* **2008**, *27*, 771–775.
72. Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T. *Curr. Neuropharmacol.* **2009**, *7*, 65–74.
73. Gilgun-Sherki, Y.; Melamed, E.; Offen, D. *Neuropharmacol.* **2001**, *40*, 959–975.
74. Singh, R. P.; Sharad, S.; Kapur, S. *J Indian Acad Clin Med.* **2004**, *5*, 218–225.
75. Schreibelt, G.; van Horssen, J.; van Rossum, S; Dijkstra, C. D.; Drukarch, B.; de Vries, H. E. *Brain Res Rev.* **2007**, *56*, 322–330.
76. Bhardwaj, P. *Trop. Gastroenterol.* **2008**, *29*, 129–135.
77. Otamiri, T.; Sjodahl, R. *Dig. Dis.* **1991**, *9*, 133–141.
78. Luciak, M. *Annales Academiae Medicine Bialostocensis* **2001**, *49*, 157–161.
79. Fryer, M. J. *Neurology* **2000**, *5*, 1–7.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

80. Gonzalez, B. *Electron J Biomed* **2003**, *1*, 5–11.
81. Al-Jawad, F. H.; Al-Atabee, H. G.; Sahib, A. S. *Pak. J. Physiol.* **2010**, *6*, 18–21.
82. Patel, B. D.; Welch, A. A.; Bingham, S. A.; Luben, R. N.; Day, N. E.; Khaw, K. T.; Lomas, D. A.; Wareham, N. *J. Thorax.* **2006**, *61*, 388–393.
83. Rahman, I. *Ther. Adv. Respir. Dis.* **2008**, *2*, 351–374.
84. Shaheen, S. O.; Sterne, J. A. C.; Thompson, R. L.; Songhurst, C. E.; Margetts, B. M.; Burney, P. G. *J. Am. J. Respir. Crit. Care Med.* **2001**, *164*, 1823–1828.
85. Guilliams, T. G. Free radicals, antioxidants and eye diseases. *The Standard* **1999**, *2*, 1–8.
86. Rhone, M.; Basu, A. *Nutrition Reviews* **2008**, *66*, 465–472.
87. Fletcher, A. E. *Ophthalmic Res.* **2010**, *44*, 191–198.
88. Pastor-Valero, M. *Gac. Sanit.* **2002**, *16* (Supl 2), 29–40.
89. Tan, J. S.; Wang, J. J.; Flood, V.; Rochtchina, E.; Smith, W.; Mitchell, P. *Ophthalmology* **2008**, *115*, 334–341.
90. Agarwal, A.; Gupta, S.; Sikka, S. *Curr. Opin. Obstet. Gynecol.* **2006**, *18*, 325–332.
91. Sikka, S. C.; Rajasekaran, M.; Hellstrom, W. J. G. *J. Androl.* **1995**, *16*, 464–468.
92. Choudhary, R.; Chawala, V. K.; Soni, N. D.; Kumar, J.; Vyas, R. K. *Pak. J. Physiol.* **2010**, *6*, 54–59.
93. Sekhon, L. H.; Gupta, S.; Kim, Y.; Agarwal, A. *Curr. Womens Health Rev.* **2010**, *6*, 84–95.
94. Eskenazi, B.; Kidd, S. A.; Marks, A. R.; Sloter, E.; Block, G.; Wyrobek, A. *J. Hum. Reprod.* **2005**, *20*, 1006–1012.
95. Ghate, J.; Choudhari, A. R.; Ghugare, B.; Ramji, S. *Biomed. Res.* **2011**, *22*, 49–51.
96. Kregel, K. C.; Zhang, H. J. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2007**, *292*, R18–R36.
97. Masaki, H. *J. Dermatol. Sci.* **2010**, *58*, 85–90.
98. Pollack, M.; Leeuwenburgh, C. In *Handbook of Oxidants and Antioxidants in Exercise*; Sen, C. K.; Packer, L.; Hanninen, O., Ed.; Elsevier Science BV: Amsterdam, Netherlands. 2000, pp 881-923.
99. Khalil, M. I.; Sulaiman, S. A.; Boukraa, L. *The Open Nutraceuticals Journal* **2010**, *3*, 6–16.
100. Tiwari, A. K. *Curr. Sci.* **2001**, *81*, 1179–1187.
101. Bandyopadhyay, U.; Das, D.; Banerjee, R. K. *Curr. Sci.* **1999**, *77*, 658–666.
102. Patel, J. M. A review of potential health benefit of flavonoids. *Lethbridge Undergraduate Research Journal.* [online] 2008, 3, Article 12. http://www.lurj.org/vol3n2.php (accessed May 1 2011).
103. Paiva, S. A. R.; Russell, R. M. *J. Am. Coll. Nutr.* **1999**, *18*, 426–433.
104. Rao, A. V.; Rao, L. G. *Pharmacol. Res.* **2007**, *55*, 207–216.

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch001

Chapter 2

Pathophysiological Implications of Altered Redox Balance in HIV/AIDS Infection: Diagnosis and Counteract Interventions

L. Gil del Valle*, Ph.D.

Clinical Pharmacology Lab, Institute of Tropical Medicine Pedro Kourí,
Autopista Novia del Mediodía, km 6 ½ , La Lisa, La Habana, Cuba
***E-mail: lgil@ipk.sld.cu**

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Oxidative stress (OS) has been detected in many tissues of HIV infected individuals using different biochemical's markers and diverse bio-techniques. The OS characterization has been made in populations from several countries and different risk groups using case and control, cross sectional and interventions studies. During the infection course the imbalance in redox status are related to oxidative molecular damage, viral replication, micronutrient deficiency and inflammatory chronic response, all of them implicated in cellular apoptosis and decreased immune proliferation. Several OS counteract actions are developed including exogenous supply of antioxidants, both as modification in micronutrient intake from diet or extract from natural origin and antioxidant vitamins. Physical exercise with a controlled program and follow- up is used too. The general strategy and combination of these interventions represent an important complementary deal for HIV infection treatment in the era of high active antiretroviral therapy. This last are involved in others associated malignancies related to mitochondrial toxicity, that in turn increase OS.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Introduction

Human Immunodeficiency Virus (HIV) infection is a worldwide increasing pandemic. HIV/AIDS patients suffer from several opportunistic infections and other malignancies that occur in relation of poor immune system function (*1*). Oxidative stress (OS), as results from the imbalance between reactive oxygen species (ROS) production and the inactivation and resolution of oxidative biomolecule damage (*2*, *3*), have been recognized since beginning of HIV infection reports (*4*, *5*). Under most circumstances, OS is deleterious to normal cell functions. An emerging view, however, is that, within certain limits, cellular redox status is a normal physiological variable that may elicit cellular response such as transcriptional activation, proliferation or apoptosis (*6*). Exposure to oxidants challenges cellular systems and their responses may create conditions that are favourable for the replication of HIV through nuclear factor κB (NF-κB) mechanism (*7*).

The hallmark of HIV infection is cellular T CD4+ depletion and dysfunction. Different agents appear may trigger apoptosis in $CD4^+$ T cell, including viral protein (i.e. gp 120, Tat), inappropriate secretion of inflammatory cytokines by activated macrophages i.e. tumor necrosis factor alpha (TNF-α) and toxins produced by opportunistic microorganisms (*8–10*). Since OS can also induce apoptosis, it can be hypothesized that such a mechanism could participate in $CD4^+$ T cell and neuronal apoptosis observed in AIDS (*11*, *12*).

Micronutrients deficiency is another aspect that contributes to OS in HIV evolution (*13*, *14*). The nutritional requirements index of the HIV infected patients are in accordance with the chronically activation of immune system. All these aspects are involved in a vicious autocatalytic cycle which underline in recurrence of malignancies, weight loss and toxicities because of exacerbate associated metabolism (*15*, *16*).

HIV Biology and Tropism

HIV constitutes the third retrovirus discovered. It is an enveloped animal RNA virus with two positive strands. It is classified as lentivirus belonging to retroviridae family. The virus genome is formed by more than 9200 base pairs longer; including the long terminal repeat (LTR) at both ends and has three major coding regions. These regions encode core (gag), polymerase (pol), envelope (env) and several “accessory” gene products (Nef, Tat, Vpr, Vif, Rev and Vpu). The accessory genes are expressed early in cell cycle infection so they modulate activation of late transcription. When Tat and Rev reach the threshold a change in frameshift reading occurs in order to express HIV constitutive genes. The generic RNA virus, codes for antigenic envelope glycoproteins, interior structural and non-structural proteins, and polymerases used for virion replication (*17*).

HIV infects cells utilizing CD4 as receptor. CD4 interact with gp120 HIV envelope glicoprotein and also needs certain chemokine receptors as coreceptors (CXR4 and CCR5). This last interact with gp41 transmembrane protein. The interactions between receptor and coreceptors guarantees HIV entry and infection. CD4+ T lymphocytes and macrophages are major target for HIV infection (*18*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Cellular tropism can be defined in terms of HIV possibility to grow in cells (examined *in vitro*). HIV isolates that grow productively in macrophages and but less efficiently in T cell lines is called M tropic. HIV which can grow in T cells but not in macrophages is classified as T tropic. HIV isolates that can grow in both T cells and macrophages are classified as dual tropic. Most of M tropic viruses utilize the CCR5 chemokine receptor and most T tropic strains utilize CXR4 receptor. The V3 region in gp120 is the major co-determinant for coreceptor use (*19*, *20*).

HIV infection is multifactor depending on biological and behavioural features of virus and host. Transmission occurs by sexual relations, breast feeding, contaminated blood products, contaminated intravenous injectors and through maternal-fetal circulation (vertical way). HIV is delivered to lymphoid tissues, spleen, lung, lives and the central nervous system (CNS). Persistence of parasitic viruses requires infecting host cells, pirating host resources, outmanoeuvring host immune components and replicating (*20*, *21*).

Progression and Surrogate Marker of Disease Evolution and Management

Although several cellular, humoral markers and viral load, which predicts progression independently of the absolute T CD4+ lymphocyte count, are reported to be associated with disease progression, only the CD4 count and viral load have come into general use because of precision and specificity diagnostic technique (*22*). In healthy adults, the T CD4+ lymphocyte counting is between 800 to 1200 units per mm^3 of blood. The T CD4+ lymphocytopenia and the increase of the viral load are parameters which determine the progression of the HIV infection which reaches its peak at the final stage called AIDS, which is the most advanced phase of the virus infection. Additional surrogate markers of progression, that add value to CD4 count, would therefore be useful in the clinical management of individual patients considering the tendency of actual anti-retroviral therapy to diminish viral load count. Highly active antiretroviral therapy (HAART) resistance and HIV mutation are affecting the prognostic value of these indexes (*22*, *23*). Also they are used during antiviral and alternative/complementary therapy evaluation (*24*).

Additional surrogate markers of progression, that add value to both, would therefore be useful in the clinical management of individual patients considering the tendency of actual anti-retroviral therapy of negative viral load. The study and refinement of surrogate markers of HIV disease progression continues to be an important area of research particularly with the advent of therapies that claim to halt or slow the process of immunological decline. Additional markers of progression would therefore be useful i.e. in the decision of when to start or change therapies.

Increased levels of CD38+/CD8+ cells have also been shown to correlate with a number of other markers of disease progression, including viral load (*24*).

The CD38 molecule is a transmembrane glycoprotein expressed at different stages of maturation or differentiation (*25*). Increased expression on lymphocytes is associated with cell activation, intracellular calcium mobilization (*26*). In CD8 cells it has been shown that the CD38+ subpopulation is in the pre-G_0-G_1 phase suggestive of a preapoptotic state (*27*). CD38+/CD8+ levels were increased with severity of HIV infection (*24*).

Human monocyte-derived macrophages are involved in a variety of pathological events during HIV infection. Even if the exact cause of the loss of $CD4^+$ T cells is unknown, the most widely accepted hypothesis is that HIV primes the cell to apoptotic death (*28*, *29*). When activated, peripheral blood T lymphocytes are induced to express Fas/APO-1/CD95 receptors that mediate apoptosis when binding to Fas ligand (*28*). A correlation between Fas expressing CD4+ cells and CD4+ T cells count in blood was observed in previous report and relationship with redox status, in agreement with the increase of Fas/CD95 found in HIV+ patients (*29*, *30*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

HIV Infection and Redox Regulation: Relation to Viral Replication and Apoptosis

Altered Redox Indexes during HIV Infection

HIV infections cause a chronic inflammation as shown by high plasma levels of inflammatory cytokines and increased ROS production in seropositive individuals (*31–33*).

Of the mechanisms contributing to the HIV progression, OS, induced by the increased production of ROS, may play a critical role in the stimulation of HIV replication, the development of immunodeficiency and the degenerative evolution of individuals (*21*). ROS generated by endogenous manner is, in certain boundaries, essential for maintain homeostasis (*2*). ROS also acts as specific signalling molecules to trigger the activation of specific signalling pathways, transmitting the effect of ROS. At low levels cellular function are regulated by a proliferative response whereas at high levels certain pathway module the cellular strategies for detoxification and thus, are essentials for survival of cell and organism. OS had in turn two main consequences: activation of specific signal transduction pathway and damage to cellular components, both of which impact on physiology and the development of diseases (*6*, *34*, *35*). Many of these effects involve activation of specific transcription factors that control the expression of a range of target genes. This encodes protein/enzymes to mediate biological response to OS.

Engagement of HIV envelope with T cell receptor meanly CD4 generate signals that lead to an increase in free intracellular calcium, which mediate protein kinase (PK) phosphorilation and activation of NADPH oxidase (*35*). In this aspect NADPH oxidase enzyme in phagocytic cell (NOX2, gp 91 ^{phox}) is critical in host defence against pathogens (*2*, *6*). This enzyme contribute to an excessive ROS production which may be related to an increased activation of polymorphonuclear leukocytes during infections or influenced by the prooxidant effect of tumor necrosis factor-alpha (TNF-α) produced by activated macrophages during the course of HIV infection (*36*)

The OS was shown to be related to the constitutive production of H_2O_2 by neutrophils at all stages of the disease, even in the early stages when the number of CD4+ T cells is still high (*36*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

To establish the implication of oxidative damage it is essential to be able to measure it accurately and in accordance with progression recognized marker of disease. Different approaches are used, sometimes measuring the levels of the damaged done by ROS and evaluating antioxidant total defences or not are taken as evidences of OS (*37*, *38*).

In Table I are summarised studies that show oxidative stressed HIV-infected populations and having significantly lower antioxidant concentrations than non HIV individuals (*33*, *39–47*). In the literature disturbs in the metabolism of glutathione (GSH), thioredoxin (TRX), ascorbic acid, tocopherol and selenium (Se), seric and tissue antioxidant diminished concentrations are reported. Increased peroxides (PO), isoprostanes (isoP), malondyaldehyde (MDA) and carbonyl (CO) concentrations and altered glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activity have been reported. In addition, altered levels were found in both pediatrics and adults HIV patients.

These findings could be explained in part by several mechanisms such as: 1-chronic inflammatory activation of immune system, 2-low intake of antioxidant or their precursors from diet in relation to requirements, 3-malabsorption, 4-enhanced cysteine metabolism in peripheral tissues, 5-down regulation synthesis of antioxidant enzymes by viral protein as Tat and, 6-virally encoded regulatory proteins. All influences on consequent loss of sulphur-group that may account for glutathione and antioxidant deficiency during HIV infection. Abnormally high levels of ROS as a consequence of chronic immune system activation by HIV infections could lead to a decline of antioxidant defence molecules and accumulative damage of cellular components generating augmented lipid peroxidation products, oxidized proteins and altered DNA sequences (*34*). Almost redox implicated enzymes and molecules are physiologically endogenous generated and are involved in detoxification and general metabolism. As a consequence of antioxidant depletion the detoxification capacity of reactive metabolites is reduced and this is probably connected to the peroxides high levels detected and drug side effects in HIV+ patients. Considering previously data OS has a dominant pathogenic action in the HIV infection (*34*).

These characteristics favours the progression of the infection with increase of viral replication, carcinogenesis, immune dysfunction, increase in the T cells and neuronal apoptosis, and disturbed patterns in cytokine and hormone production (*2*, *33*). Thus, the clinic significance of the OS related with the HIV is reflected by the strong association between decrease of survival of these individuals and increase of oxidative damage indexes in the plasma and the CD4+ lymphocytes.

Other experiments suggesting that lipid peroxidation is much more important in the asymptomatic stage rather than in AIDS. An explanation for this may relate to the depletion of neutrophils which occurs in the late disease stages and which may be influenced by the treatments used by patients too, so increased damage indexes values may be a consequence of the multifactorial nature of the redox system (*21*, *23*).

Peroxides serve as a source for hydroxyl or peroxyl reactive radicals who can interact with cellular components inducing cell damage potentially leading to cell death (*2*, *6*). The increase of PO observed in HIV+ patients emphasizes the higher OS, which occurs during HIV infection. It should also be noted that peroxides and aldehydes generated are not only passive markers of OS, but also cytotoxic products (*38*). It is thus important to evaluate the role to these oxidative products in lymphocyte death. Nuclear DNA fragmentation is a biochemical hallmark of apoptosis (*2*, *3*) and its increase in HIV+ patients. The pathology of ROS is related to oxidation of nucleic acids and chromosomes break. Limited chromosomal damage can be repaired whereas extensive DNA damage promotes apoptosis (*8*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Table I. Evidences of biomolecule oxidative damage and antioxidant deficiency in HIV/aids patients

PLACE	*NI*	*EVALUATION CRITERIA **	*REFERENCES*
Grenoble (France)	43	P Gluthatione , Malondialdehyde , Total peroxides	Favier *et al.* (1994)
Buenos Aires (Argentina)	20	P Gluthatione, Total antioxidant capacity, E Superoxide dismutase	Repetto *et al.* (1996)
Bonn (Germany)	102	E Gluthatione, S Gluthatione and Selenium	Look *et al.* (1997)
Stanford (USA)	204	P Gluthatione	Herzenberg *et al.* (1997)
Toronto (Canada)	29	L Gluthatione and Cistein	Walmsley *et al.* (1998)
Toronto (Canada)	49	P Total peroxides , EA Ethane	Allard *et al.* (1998)
La Habana (Cuba)	85 (Adults) 11 (Child)	P Gluthatione peroxidase (-), E superoxido dismutase (-), P Malondialdehyde (+), P hidroperoxide (+) P Total Antioxidant capacity (-), P Gluthatione (-) L percent of DNA fragmentacion (+)	Gil *et al.* (2003) Gil *et al.* (2002)
India	50	S Total antioxidant capacity (-), Malondialdehyde (+), Superoxide dismutase (-), vitamin E and C (-)	Suresh *et al.* (2009)
Italy	26	P hidroperoxide (+), P Total Antioxidant capacity (-), P thiol group (-)	Coaccioli *et al.* (2010)

Legend NI: Number of individuals. *Statistical analysis with significant difference respect control group ($p<0.05$). – diminish ↔ no change + increment. P- plasma, S- serum, L- lymphocytes, EA-expired Air, E-erythrocytes.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Antioxidant enzymes levels are sensitive to OS. Both increased and decreased levels have been reported in different disease states in which an enhancement of ROS could be a cause or a consequence of the disease. Several kinds of molecules contribute to the antioxidant capacity of plasma. The possible interaction among different antioxidants *in vivo* could also render the measurement of any individual antioxidant less representative of the overall antioxidant status (*38*). There is thus experimental evidence that recognize different metabolic events that occur as a consequence of HIV infection directly influence the consumption of antioxidant components thus contributing to the OS (*34*).

The behaviour characterization of total antioxidant capacity and other indexes in HIV patients point to the multifactorial feature of this infection. Some authors consider OS not as an epiphenomenon, but rather as having a central role in HIV disease (*34*).

These revised studies provide evidences of oxidative damage to different molecules corresponding with diverse clinical conditions in relation to CD4+ lymphocytes subset and viral load. The combination of redox indexes that could be most useful based on that is not clear but the behavior and correlation with progression markers could be contributing in follow up evaluations.

HIV Replication and Oxidative Stress

In HIV-infected patients OS has been implicated in increased HIV transcription through the activation of NF-κB (*36*). NF-κB is bound to factor I κB in the cytoplasm in its active form, but various factors, such as TNF-α and ROS can cause the release of NF-κB from factor IκB, and NF-κB translocates to the nucleus and binds to DNA. GSH is a major intracellular thiol, which acts as a free radicals scavenger and is though to inhibit activation of NF-κB (*36*). TRX is related to redox regulation of IκB. NF-κB is involved in the early transcription of HIV-1. Thus, ROS may potentially be involved in the pathogenesis of HIV infection not only through direct effects on cells instead off through interactions with NF-κB and activation of HIV replication too.

Some viral proteins interfere with host T cell functions and promote rampant virus replication. Taylor *et al.* (1997) pointed out several regions of HIV-1 with the potential to encode selenoproteins, a GPx viral homologue and a thioredoxin reductase (TDR) homologue. These could be contributing to Se host deficiency. Significance of host Se status is based on the antioxidant properties of amino acid selenocysteine, the catalytic center of selenoenzymes as GPx family (*48*). This enzyme regulates biologic oxidative homeostasis by neutralizing metabolically produced ROS (*3*, *49*).

The viral selenoproteins have been suggested could be involved in regulation of NF-κB controlling HIV replication (*35*, *48*). TDR homologue viral expression could be contributed to activation of NF-κB while reducing cellular GPx levels via Se sequestration. Whereas the viral GPx homologue- as a late expressed gene- would be expected to deactivate NF-κB by decreasing oxidant tone. Notable another TDR viral homologue action is in the synthesis of deoxyribonucleotides, via ribonucleotide reductase, enhancing stimulation of proviral DNA synthesis activities (*49*).

These dietary and antioxidant Se-deficient nutritional conditions found in HIV individuals contribute to the physiologic OS conducive to impaired immune systems and RNA viral mutations, which can be virulent and pathogenic (*49*, *50*).

Translation initiation of the HIV' full length messenger RNA is suggested through cap-dependent but also use an internal ribosome entry site (IRES) located in the 5′ untranslated region. This site are RNA structure region that directly recruited the 40s ribosomal subunit at or near an initiation codon. Various models for the 5′ UTR encompassing IRES have been proposed based on phylogenetic, chemical mapping and mutagenesis approaches. The wild type compose include stem loop. Translation process requires host cell factor called IRES trans-acting factor (ITAF). Researches concerning *in vitro* experiments strongly argue that OS in HIV increase the IREs activity via an effect on stem loop. This data combines chemical and conformational changes caused by OS in favor of viral replication (*51*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Apoptosis during HIV Infection in Relation to Oxidative Stress

In vivo studies have shown that OS might lead to immunodeficiency at cellular and humoral levels. The role of OS in lymphocytes depletion during HIV infection may result from different mechanism such as impairment of proliferation, as suggested in animal models, but also from apoptosis (*8*, *9*, *12*). Several investigators have proposed that apoptosis, could be initiated by OS, and is the direct cause of lymphocyte loss in patients infected with HIV (*10*, *29*). These investigations provide evidence that infection-induced OS contributes to CD4+ T lymphocytes depletion by increasing their rate of apoptosis, particularly Fas/APO-1/CD95 induced apoptosis. The pro-oxidant stimuli can also increase the pathogenic effects of HIV and is associated with a progressive increase of plasma viral load too (*28*).

CD4+ T cell subset depletion in HIV/AIDS patients is the most dramatic effect of apoptosis mediated by redox abnormalities and induction of Fas/APO-1/CD95 receptor expression (*30*). The proportion of lymphocytes expressing Fas was shown to be elevated in HIV –infected individuals. Generally these studies demonstrated that the proportion of Fas-expressing cells increases with diseases progression, the increased Fas expression was found by some investigators to be in CD4+ lymphocytes and by others in both CD4+ and CD8+ T cells (*29*).

In previous work, it has been demonstrated that viral Tat protein liberated by HIV-1-infected cells interferes with calcium homeostasis, activates caspases and induces mitochondrial generation and accumulation of ROS, all being important events in the apoptotic cascade of several cell types.

HIV allocation in central nervous system (CNS) induces progressive multi symptoms of motor, cognitive dysfunction and behavioral changes. The neurological and psychiatric characteristic caused by HIV infection is referred as associated dementia (HAD). The *in vitro* and *in vivo* experiments suggest that neuronal apoptosis occurred in absence of direct infection (*12*). Activated microglia and macrophage infected or not produce proinflammatory cytokines (TNF, IFNγ, Il-1β, arachidonic acid), inducible nitric oxide synthase, quinolate, PAF, glutamate and quinolic acid. This products as viral proteins promote neuronal toxicity via the NMDA type glutamate receptor. The receptor activation and ROS increased generation may initiate caspase activation or promote calcium influx. Also dopaminergic system may be involved in HAD. The recognized indirectly pathway are attributed to neurotoxins release by activated cells in the CNS producing excitotoxicity and OS. This involving viral proteins too (*52–54*).

Observations showed that free ROS-induced apoptosis by a non cytokine/ mediated mechanism was significantly enhanced in HIV-infected subjects even in the very early stages after infection. Moreover, ROS-mediated apoptosis was not restricted to a particular cell.

HIV Infection, Nutritional Requirements, and Micronutrients Deficiency

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Although major advances have been made in understanding the biology of HIV infection and significant progress in therapy has been carried out in the last few years (*55–59*), the basic role of host nutrition in the pathogenesis of HIV remains a major lacuna in the scientific knowledge (*60*).

Micronutrient deficiency is often common during the HIV infection. Insufficient dietary intake and increased requirements contribute to the development of micronutrients deficiency (*1*, *16*, *61*). The premise that micronutrients deficiency plays a role in the pathogenesis of HIV infection is mainly based on two theories, i.e. the OS theory and the nutritional immunological theory, both of which have related and overlapping mechanism (*3*).

It is possible that prolongation of the interval between infection and symptoms observed over the last decade might relate to HAART but in part to better nutrition too (*1*, *60*, *61*). That possibility should encourage additional studies of nutritional status because controlled interventions (in particularly high doses of antioxidants) might determine whether nutrition can remedy the possible consequences of abnormal nutritional status and how it can be influence on adverse reaction of HAART (*60*).

Some micronutrients play essential roles in maintaining normal immune function and its may protects immune effector's cells from OS (*2*). For HIV patients is particularly important to identify metabolic alterations and nutritional deficiencies and determine whether the supplementation will improve clinical outcome. This requires realistic and sustainable healthcare interventions in terms of costs, technology transfer and independence from sophisticated monitoring requirements.

Early in the HIV epidemic was observed a relationship between loss of lean body mass and timing of death in patients with AIDS (*55*). Such an impact of nutritional state on survival was shown by Suttmann *et al.* (1995), who demonstrates an independent effect of loss of body cell mass in survival (*57*). If malnutrition does accelerate the progress of HIV infection, it might be argued that improving nutritional state may improve clinical outcome (*34*).

The impact of both individual and diverse micronutrients on HIV disease has been studied by several groups (*13*, *15*, *46*, *50*, *58*, *59*, *62–65*). Baum *et al.* (1995) showed an association in development of vitamin deficiency with reduction in T

CD4+ cell count and conversely the normalization of vitamin concentrations was associated with an increase in T CD4+ cell count (*15*). Some investigators have chosen to look at micronutrient intake rather than plasma levels because these may not be the most sensitive indicator of micronutrient status due to metabolic processes depress the plasma concentrations of some of them (*14*). Tang *et al.* (1993) demonstrated that high intakes of vitamin C, thiamin or niacin were associated with a reduced relative risk of disease progression (*13*). Vitamin A intake appears to have a bi-modal shaped relationship with outcome, both very high and very low intake being associated with poorer outcome (*56*).

During the HIV infection the nutritional requirement are modified and are not in accordance with the conventional RDA. Even when in HIV patients a significant increment in the intake of vitamin A, E and C was noted, those values do not reach the critical (satisfied <70% of the individual requirements) established by WHO (*57*, *61*, *66*).

Elevated lipid peroxidation products (MDA, PO), damage to DNA and depleted antioxidant capacity (TAC, GSH, GPx) have been documented in HIV populations in relation to micronutrient deficiency and provide additional evidence that individuals with HIV infection develop moderate to severe micronutrients deficiency and that deficiency could be related to their redox status (*61*). These conditions could be associated with cell damage, metabolic abnormalities and immune dysfunctions all of which are observed in HIV/AIDS patients. Increment in micronutrient intake but unaltered energy and macronutrient intake study noted a significance increase in antioxidant status evaluated by an increase in TAC, GPx and GSH and a decrease in SOD (*63*).

Several kinds of molecules could contribute to the antioxidant capacity of plasma from exogenous or endogenous origin. An association between vitamins A, E, C, vegetables and fruits intake, minerals and total antioxidants status suggests a role of antioxidant-pro-oxidant balance on T cells stabilization and functions which resound in immune response. The mechanisms underlying the present observations are not clear but multiple pharmacological effects of antioxidant vitamins and flavonoids have been reported, including vascular protection, anti-inflammatory, anti-tumor and anti-hypertension activities (*3*).

Flavonoids also posses free- radicals scavenging abilities, and their anti-radical property is directed towards both hydroxyl radicals and superoxide anions both of which are implicated in the initiation of lipid peroxidation. Only a limited number of studies on the antioxidant action of flavonoids at the cellular level have been reported and little is known about the mechanism of the cellular reactions (*2*, *67*, *68*). Flavonoids can however prevent oxidative cell damage by either increasing glutathione, which is indispensable for the peroxides reducing activity of GPx or protecting cell glutathione depletion with the cooperation of ascorbic acid (*61*).

Actual analysis of nutrition influence of HIV infection evolution suggest that the evidence-base for the specific effect of micronutrient supplements in children and adults infected with HIV is limited, but is sufficient to make some recommendations for patients management (*60*). In the absence of population-specific adverse effects, there is no reason to decline similar recommendations for HIV-infected populations. In general order the individual

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

or combined vitamin supplementation are encourage in keeping with WHO2003 recommendations (*66*), everything possible should be done to promote and support adequate dietary intake of micronutrients at Individual Nutrient Intake Level (INL98) levels, while recognising that this may not be sufficient to correct specific micronutrient deficiencies in all HIV-infected individuals.

The optimal composition and dosage of various supplements requires investigation, as these can vary considerably among commercial supplements and therefore may not have equivalent effects Research participants should be diverse with respect to stage of disease, use of antiretroviral therapy, immune status, and nutritional status.

It is important during the supplementation recognize between the micronutrients administration as nutritional factor for immune response and the pharmacological use of high doses of them as preventive therapy in HIV infection. A recent controlled trial demonstrated that multivitamin supplementation delayed the progression of disease among HIV-infected women. In the light of these observations it becomes advisable the administrations of multivitamins, except vitamin A in HIV infected persons before the initiation and during antiretroviral therapy.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

HIV Infection, Redox Regulation, and HAART

When patients are diagnosed as HIV+, antiretroviral therapy is initiated when CD4 + T cell subset fall bellow 200 lymphocytes /mm^3, when HIV infection is fairly advanced. The combined therapy named High Active Antiretroviral Therapies (HAART) applied varies depending on therapeutics objectives, cost and availability on the market. In the latest years, a relevant decline of the morbidity and mortality of HIV infection has been observed due to the use of HAART (*69*, *70*). HAART have led to a decrease of viral load, and a quantitative and qualitative improvement of the immune functions in patients, specially T-CD4+ lymphocyte count, having as a consequence a decrease of infectious complications and a global clinical improvement (*69*).

The HAART therapy consist of different combination of viral inhibitor such as: nucleoside reverse transcriptase inhibitor (NRTI), non nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), fushion inhibitor (FI), co-receptor inhibitor (CRI) and integrase inhibitor (INI). The main reasons for combining different anti-HIV drugs in the treatment of AIDS as (i) synergy, (ii) lower toxicity and (iii) prevention of drug resistance development have remained (*69*, *70*).

But HAART does not completely solve the immune and metabolic alterations during HIV evolution. Instead hepatic toxicity from AZT, ddI, and ddC was reported early in the epidemic. Recent reports continue to point at the mitochondria as toxic target and activation of the P450 hepatic system by PI (*71*).

Mitochondrial toxicity as an important side effect of antiretroviral therapy in HIV infection is linked pathophysiologically to clinical and experimental settings with long-term significance (*72–77*). Combinations of anti-HIV drugs containing NRTI are used during HIV infection evolution as clinical guidelines recommend

(*70*). Such regimens may be important to the development of mitochondria toxicity. Clinical and basic evidences for HAART mitochondrial toxicity in relation to energy deprivation secondary to mitochondrial (mt) DNA depletion, oxidative stress which produced structure damage and altered functions, and mtDNA mutations resulting from oxidative mtDNA damage, aberrant mtDNA replication and altered mtDNA transcription are reported (*74–76*).

Essential Elements of Mitochondrial Functions

Mitochondria are semi-autonomous organelles that perform essential functions in cellular metabolism and the regulation of cell death. It is widely believed that they arose from an endosymbiotic relationship between a glycolityc pro-eukaryotic cell and an oxidative bacterium (*78*, *79*). Mitochondria posses a double-membrane structure and contain their own genome along with their own transcription, translation, and protein assembly machinery. As such, they are able to maintain genomic independence from the nucleus. However, most proteins in the organelle are encoded by nuclear DNA (nDNA) and imported into it. Although DNA mitochondrial replication (mtDNA) is not synchronized with nDNA replication, the overall number of them per cell remains fairly constant for specific cell types during cellular events (*76*). It suggests that the generation of mitochondria is mainly influenced by extra-mitochondrial transduction signal (*74*).

The most well-known and best-characterized function of mitochondria is the production of adenosine triphosphate (ATP) through oxidative phosphorylation (*80*). The process is carried out by the respiratory chain which contains 87 polypeptides encoded by both mtDNA and nDNA. This result the unique biochemical process achieved by a well coordinated effort of the products from two separate genomes (*81*).

Glycolisis can also generate ATP and provides a compensatory mechanism when the phosporylation become inefficient as a consequence of defect in respiratory chain (*80*, *81*).

The normal electrons flow could be compromises as a consequence of certain mtDNA mutation. It can lead to an increase of bifurcation and ROS generation. Mitochondria are indispensable to eukaryotic cells due to their extensive involvement in critical metabolic processes (*76*, *81*, *82*).

Mitochondrial Dysfunction Hypothesis during Antiviral Treatment

DNA polymerase-γ (DNA pol-γ) is the eukaryotic mtDNA replication enzyme. It is encoded by nDNA and contains two subunits. The DNA pol-γ activity is inhibited by (NRTI) used commonly during antiviral treatment of HIV/AIDS patients. This inhibition produces mtDNA depletion which leads to a decreased energy production. This event is cumulative and toxic manifestations increase with duration of exposure (*76*, *81*).

NRTI have been divided into classes of mtDNA replication inhibitors according to the relative importance of DNA chain termination, or internalization of the analogue into nascent mtDNA and substitution for the natural base.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Competition with the native nucleotide and NRTI at the nucleotide binding site of DNA pol-γ appears to be a crucial event (*83*).

In the model of competitive inhibition fialuridine monophosphate (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil; FIAU) is a representative. Others NRTI which share this characteristic are FIAC (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodocytosine), FMAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-methyluracil) and FEAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-ethyluracil). Each has demonstrated virucidal effects in disease models. The second type of NRTI is represented by dideoxynucleotides: 3′ –azido-3′–deoxythytmidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′- dideoxycytidine (ddC), (-)-2′-deoxy-3′-thiacytidine (3TC), 2′,3′ –didehydro-3′-deoxythymidine (d4T) which compete with natural nucleotides as above but it also terminate nascent mtDNA synthesis because they lack 3′-hydroxyl group (3′-OH) for continued mtDNA polymerization. Accordingly, these compounds may have potentially hazardous consequences (*83*–*85*).

Deletion mutants (truncated mtDNA templates) may be replicated more quickly and efficiently than native mtDNA counterparts owing to enzyme activity. Abundance of defective mtDNA may reach a threshold of energy depletion like that of heritable mitochondrial illnesses, including those that show mtDNA depletion (*86*–*92*).

Mitochondrial dysfunction hypothesis incorporated the inhibition of DNA pol-γ, OS occurring and acquired mtDNA mutations into a pathophysiological continuum related to energy depletion in HIV/AIDS patients with NRTI treatment (*80*, *84*, *87*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Oxidative Stress during HIV Infection and NRTI Treatment

Inhibition of DNA pol-γ by NRTI contributes to both an altered expression of proteins component of respiratory chain and an increase in bifurcation of electron flow mainly at complexes I and III. They may also lead to the dysregulation of the mitochondrial enzyme systems (inhibition of succinate transport or cytochrome oxidase activity, reduction in carnitine). This implicates an increment in mitochondrial ROS generation and OS recrudescence. OS may amplify some of pathophysiological and phenotypic events in NRTI toxicity. Mainly OS are implicated in mutations of mtDNA based on: (1) lack of known repair enzymes for mtDNA error excision; (2) lack of histones protecting mtDNA, and (3) a subcellular proximity of mtDNA to oxidants (*81*, *93*).

Oxidative Stress in Mitochondria and Mutations of mtDNA in NRTI Toxicity

The results of these events are extensive strand breakage and degradation of deoxyribose. The oxidative mtDNA mutations may be by peroxynitrite and hydroxyl radicals would likely inactivate complex I first which could amplify ROS generation and increase OS (*87*, *93*).

Hydroxyl radical's oxidation to mtDNA results in formation of oxidized base 8-hydroxydeoxyguanosine (8-OHdG). This in DNA leads to GC → AT transversions unless repaired. It can lead to mispairing and point mutation.

Although most of the components of a mitochondrial base excision repair system have been identified, it is unclear how efficiently this repair removes the wide sprectrum of adducts that may occur from oxidative damage. Mitochondrial oxidative damage was supported indirectly by the co-existence of MDA on or near the inner mitochondrial membrane. This last could lead to cross-linking, deletion errors in transcription or mtDNA polymerization (*74*, *76*, *82*).

An accumulation of mtDNA defects may result in myocytes with oxidative capacity that varies from normal to severely impair in a so-called myocardial bioenergy mosaic in the NRTI treated cells (*83*). Pathophysiological events not will occur until the thresholds of damage were severe enough to impact on organ function (*2*, *3*).

Clinical Evidences of NRTI Mitochondrial Toxicity and OS

From early in the era of HAART, spectrums of changes in body fat have been reported to occur in 20%-80% of subjects receiving these therapies (*88–92*). In those with abdominal obesity, hypertension, abnormal serum lipids, and evidence of insulin resistance, this constellation simulates features of the dysmetabolic syndrome (syndrome X) (*91*). Moreover, there is also evidence that the loss of subcutaneous fat *per se* in persons with HIV is associated with insulin resistance (*92*).

Extracts of muscle biopsy specimens of AZT treated had depleted skeletal muscle mtDNA. Inefficient used of long-chain fatty acid for β-oxidation occurs and fat droplets accumulate (*94*).

A high lactate/piruvate ratio (consistent with abnormal mitochondrial function) was detected in the blood of patients with HAART toxicity (*95–99*). Controversy exists regarding potential mechanism. Although extensive experimental data may lack to support the hypothesis, it is reasonable to consider the possibility of subclinical mitochondrial dysfunction and resulting anaerobic metabolism may relate to this observation (*72*, *83*, *87*).

The experimental literature show evidences of mitochondrial changes in selected tissues from rats, mice, other rodent species and primates with a variety of NRTI dosing schedule (*100–102*). Even current human therapeutic doses or NRTI doses that are below those currently used clinically, mitochondrial defects are demonstrated in tissues. Respect to mitochondrial toxicity of NRTI, cardiomyopathy (CM) was reported by many authors in relation to AZT and /or other NRTI. CM is reported in AIDS, but is remains controversial (*86*, *88*, *94*, *98*). Features of AIDS CM are shared with the documented AZT myopathy. Clinical features of AZT CM occur after prolonged treatment.

Hepatic toxicity from AZT, ddI, ddC, d4T and 3TC was reported early in the epidemic (*103–112*). Fatal hepatomegaly with severe steatosis, severe lactic acidosis and adult Reye's syndrome in AZT - treated HIV seropositive patients were all pathogenetically linked to AZT-induced hepatotoxicity. Clinical features resembled some of those seem in FIAU toxicity (*85*).

Treatment with certain NRTI (d4T/3TC combinations) results in anion gap acidosis (*103*). Moreover the lactic acidosis/hepatic steatosis syndrome may be more common than previously appreciated in adults and children treated with

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

NRTI and d4T has been suggested as a culprit in these cases. d4T treatment cause lipodystrophy (*96*, *104*). This last phenotype is observed in patients long treated with others NRTI (*104–108*). Lypodystrophy consist of two components that may be analyzed together or independently: fat accumulation and fat atrophy. Lipodystrophic syndrome in HIV-1 infected patients is characterized by an abnormal distribution of fat tissue with subcutaneous lipoatrophy of the face, arms, and legs, and an increase in visceral fat of abdomen and back of the neck or by subcutaneous fat loss in the arms and legs without accumulation of abdominal fat (lipoatrophy). Fat accumulation is seen within the abdominal cavity, the upper back (dorsocervical fat pad), the breast, and in subcutaneous tissue. Fat atrophy is ascribed to d4T, lesser extent to AZT and ddI (*107*, *108*). Mechanism may involve altered mitochondrial biogenesis and/or oxidative changes, and possibly adipocyte apoptosis. Adipose tissue is now recognized as a main site for the release of hormonal signals that influence CNS, peripheral tissues and the subsequent alteration in overall metabolism. A meta-analysis of 5 series with 5435 HAART recipients showed fat accumulation in 9% to 34% and fat atrophy in 12% to 40% (*107*).

These rates of lipodystrophy are lower (12%-40%) than those reported earlier by Carr *et al.* (1999) (83%) and emphasize differences in methodologies, potential for selection bias, and the need for reproducible case definitions (*108*). Although the mechanisms involved in the onset of lipodystrophy are still unknown, mtDNA depletion has been observed in peripheral adipose tissue, peripheral blood mononuclear cells (PBMCs), and skeletal muscle of lipodystrophic patients (*81*).

To date, no effective therapies are available to reverse HAART-related lipodystrophy, although switching to a stavudine-, zidovudine-, or NRTI-sparing regimen seems to be the best option (*109*).

Mitochondrial toxicity from NRTI may impact on peripheral nervous system function and as the epidemic continues, these side effects are increasingly important. Dose-relating painful peripheral neuropathies occurred in the majority of patients treated with ddC in doses of 0.03-0.09 mg/kg per day. Peripheral neuropathy occurred in 55% of d4T treated patients after 1 year treatment. Clinical distal dysesethesias, areflexia, distal sensory loss, and mild muscle weakness were common. Sural nerve biopsies for patients with ddC neuropathy showed axonal degeneration and mitochondria with disrupted cristae. 3TC has an associated peripheral neuropathy (*110*, *111*).

Attenuation and prevention of these effects could improve efficacy of HIV treatment and it will resound in HIV subjects' quality of life.

Since different groups have tested the symptom effects of HAART in these populations it was therefore appropriate to test the effect of HAART on surrounded marker as mitochondrial toxicity and OS indexes during progression of treatment and infection (*112–123*). Authors assessed OS indexes in HIV + individuals before HAART treatment show altered indexes after period under treatment (Table II). Works about HAART influences on OS indexes noticed a decrease in the antioxidant system, an increase in damaged molecules and a failure to repair oxidative damage. These results are controversial with some authors but consistent with others.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Table II. Evidences of biomolecule oxidative damage and antioxidant deficiency in HIV/aids patients with HAART

PLACE	*NI*	*EVALUATION CRITERIA **	*REFERENCES*
EEUU	120	3 combinations F2 isoprostanes (+)	Hulgan T *et al.* Clin Infect Dis J (2003)
Norway	20	2 combinations MDA (-), GSH (-), GSSG(+), VIT E (+), VIT C (-)	Aukrust P *et al.* Clin Infect Dis J (2003)
EEUU	164	2 combinations Syndrome metabolic X, oxidative stress indices (+) associate to PI ± 6 months	Hurwitz B *et al.* Cardiovascular Toxicology (2004)
Cameroon	85	3 combinations Groups sulfhydrils (-), malondialdehide (TBARS) (+), carbonyls groups (+), albumin (-) y Vit C (-) in plasma	Ngondi J *et al.* AIDS Res Ther (2006)
EEUU	164	1 combination ddI/d4T (lipodistrophy symptons) 75 cases, 71 control F2 isoprostanes (no association)	Hulgan T *et al.* J Acquir Immune Defic Syndr (2006)
Spain	245	2 combinations (NNRTI, PI) total peroxide concentration (+). PI 2-month	Masiá M *et al.* Clin Therap (2007)
Italy	86	3 combinations Serum total antioxidant capacity (d-ROMs) (+) and antioxidant activity (OXY-adsorbent as MPO activity) (-) related to treatment adherence	Mandas A *et al.* J Biomed Biotechnol (2009)
India	100	3 combinations LPO, GSH ↔ PI 9,2 ± 7 months	Wanchu A *et al.* AIDS Res Hum Retrov (2009)
EEUU	194	4 combinations AZT/ d4T/EFZ/ NVP Plus 91 control F2 isoprostanes (no association with HIV-1 RNA or HAART)	Redhage LA *et al.* HIV Clin Trials (2009)
Cuba	56	2 combinations MDA (-), GSH (-), PP, HPO, CAT, SOD, PAOP in plasma PI ± 6 months	Gil *et al.* Biomed Aging Path (2011)

Legend NI: Number of individuals. *Statistical analysis with significant difference ($p<0.05$). – diminish ↔ no change + increment.

Ngondi *et al.* (2006) assessed the effect of different HAART in OS parameters and found an increase in lipid oxidation while antioxidants decrease. They did not found differences related OS damage with antivirals combination. They showed significant differences in lineal association between some parameters as sulphydryls and albumin with CD4+ T lymphocytes count and with viral load (*117*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Hulgan *et al.* (2003) quantified plasma F2 isoprostanes as OS index. They planned different strategies and found that this index augmented in 120 HIV+ individuals treated with HAART but they could not distinguish differences due to drugs or HAART combinations (*114*). During long term follow-up they did not found association with peripheral neuropathy or cardiovascular risk (*116*) in their studies context. Meanwhile Masia *et al.* (2007) found increased plasma peroxide associated with established cardiovascular risk factors in the study context (*119*).

Several studies relating OS with HAART associated to mitochondrial dysfunction were published (*74*, *86*, *87*). Although the relation is suggested there is still a debate about whether it plays a direct role in HAART toxicity. The mechanism is unclear. DNA pol γ inhibition favours to an energy deprivation and increase ROS formation. OS-mediated cell damage results in part via ROS reactions (*87*) mainly due to NRTI. This antiviral class has been implicated as a cause of several insidious and sometimes irreversible chronic toxicities.

Gil *et al.* (2011) shows redox indexes at baseline in both groups of treatment were similar (*123*). Those parameters values were different than those reported previously probably because the methods used. Observations permit to assume that HAART is responsible of the OS values change taking in to account that it was the only change introduced with a good compliance. Observation remarks that both combination used produced an increase in OS indexes paralleled to HIV progression marker change and BMI. The significant differences found in biochemical and clinical values due to HAART show worse prognostic for the D4T, 3TC, NEV combination.

In the study was demonstrated no significant linear relations for the majority of declared variables for both therapy combinations. However, significant linear regressions were found for PP- CD4+ T lymphocytes, MDA-VL, PO-VL and GSH-VL in therapy I and MDA-VL, HPO-VL and GSH-VL in therapy II. In a previous work a relationship between PP and CD4 + T lymphocytes were found in HIV patients' naive of treatment (*33*) which persisted in therapy I group but was lost in the other group.

A beneficial change in CD4+ T cell count and viral load were observed in about 80% for both treated groups. These are in accordance with the HAART therapeutically effects reported (*1*, *70*), but when redox indexes are considered in simultaneous variable analysis, significant differences ($p<0.05$) appear between therapy combinations comparisons, indicating a worse prognostic for therapy II. Therapies I and II showed different magnitude in second simultaneous change variable which presents 60% failure associated to toxic metabolic alterations related to OS. Differences could be achieved to the manner and accumulative impact on redox toxicity by each individual antiviral drug as well as the combinations (*77*).

Previous works describe some clinical and metabolic benefits during replacement of antiviral treatment. Lipatrophy, as consequence of antiretroviral treatment, was improved recently replacing d4T (used for > 3 years) with abacavir or AZT in 16 HIV individuals during 48 weeks. For that mitochondrial indices and fat apoptosis were evaluated. This result indicates that antiretroviral associated mitochondrial toxicity during HIV infection could be reversed (*109*) while others found it during dietary micronutrient intake improvement in treatment naive patients (*63*, *124*).

Theoretical an OS enhance occurs as a dynamic process of immune reconstitution too (*90*). In most of HIV patients the treatment aiming to reduce and control viral replication could produce clinical deterioration as newly inflammatory process occurs when HIV becomes a chronic condition. Immune reconstitution is related to lymphocytes redistribution, systemic immune activation changes, modification of lymphocytes turnover rate, and others events (*125–127*). This hypothesis will require larger prospective studies including immunological assessments. Long term clinical implications of OS and how it is related to antiretroviral therapy associated disease should be studied.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

HIV Infection and Antioxidant Supplementation

Since antiretroviral therapy does not completely eliminate HIV, it is likely that the final outcome of treatment will depend not only on the efficacy of treatments in reducing viral load, but also on the immune system ability to recover and control residual virus. Date showing oxidative damage together with results pointing to the ROS-mediated activation of the virus, strongly argue in favor of the benefits of early antioxidant therapy (*34*, *69*, *74*). If oxidative damage contributes significantly to the HIV infection then actions that decreased it should be beneficial for infected individuals. Taking into account that the antioxidant actions have to be designing in order to not remove totally ROS, but control their levels so as to allow useful functions whilst minimizing oxidative damage.

The redox indexes follow up could be contributing to an integral overview in HIV infection; in addition further studies may be justified to evaluate the role of ROS as apoptosis mediators and as indexes of treatment efficacy.

It seems therefore logical to try to restore appropriate redox balance during HIV disease, meanly restoring antioxidant deficiencies (*34*). This notion becomes even more attractive, when one takes into account that a wide variety of antioxidant, including GSH replenishment have been shown to inhibit HIV replication *ex vivo*. Alleviating wasting and improving liver function may also the beneficial effects of this treatment. GSH deficiency has been associated with impaired survival in HIV disease (*43*).

Table III show several studies with limited beneficial results in antioxidant and vitamins interventions (*42*, *62*, *64*, *65*, *128–137*).

N-acetylcysteine administration have been elucidated that contributes to replenish GSH in HIV patients and suggesting it can improve their survival (*42*).

Others researchers have been evaluated the effects of vitamin E and C supplementation during 3 months on these patients. They showed that this combination of antioxidant vitamins could reduce OS and produce a trend towards a reduction in viral load (*134*).

Intakes of thiamine, riboflavin, and niacin were positively associated with T CD4$^+$ cell count and inversely associated with HIV disease progression among men in the San Francisco Men's Health Study (SFMHS) cohort.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Table III. Control studies evidences of nutritional intervention and antioxidant supplementation in HIV/aids patients

PLACE	*GROUP*	*NI**	*INTERVENTION*	*EVALUATION CRITERIA ***	*REFERENCES*
Durban (Sudafrica)	Childs	28	Vitamin A doses accordin to age	(-) diarrhea	Coutsoudis *et al.* (1995)
Portland (EE UU)	Adults	72	β-carotene 180 mg	↔T CD4+ lymphocytes	Coodley *et al.* (1996)
Korea	Adults	16	Red Ginseng korean 5,4 g daily during 4 years	(+)T CD4+ lymphocytes (-)T CD8+ lymphocytes	Cho *et al.* (1997)
Bonn (Germany)	Adults	24	Selenium 500 mg + N acetylcysteine 600 mg daily during 6 months	(Tendency to +) T CD4+ lymphocytes Viral Load ↔	Look *et al.* (1998)
Cape Town	Childs	75	Vitamin A 60 mg	(-) T CD4+ lymphocytes	Hussey *et al.* (1998)
Dar Es Salaam (Tanzania)	Pregnant Womens	1075	Multivitamins + Vitamin A/ β-caroten, Vitamin A/ β-caroten	(-) fetal mortality and low weight borne	Fawzi *et al.* (1998)
Toronto (Canada)	Adults	49	Vitamin C (1g) and E (800 mg) daily during 3 months	(-) lipd peroxidation (-)viral load	Allard *et al.* (1996)
Italy	Adults	35	Zinc Sulphate 200 mg daily	(-) morbility	Mocchegiani *et al.* (1995)
Stanford (EE UU)	Adults	246	N acetylcysteine (2,3-8,8 mg) daily during 8 months	(+) GSH (+) quality of life (2 years)	Herzenberg *et al.* (1997)
Stanford (EE UU)	Adults	81	N acetylcysteine (8 mg) daily during 8 weeks	(+) GSH	De Rosa *et al.* (2000)
La Habana (Cuba)	Adults	81	*Mangifera indica* (2,4 g) daily during 6 months	(+)GSH, TAC, (-) GPx , OP, MDA, SOD, %FADN	Gil *et al.* (2002)

Continued on next page.

Table III. (Continued). Control studies evidences of nutritional intervention and antioxidant supplementation in HIV/aids patients

PLACE	*GROUP*	*NI**	*INTERVENTION*	*EVALUATION CRITERIA ***	*REFERENCES*
Canada	Adults	331	Multivitamins (incl. vitamin A and trace elements) + natural mixed carotenoids (equiv to 120000IU beta-carotene daily) four capsules daily for mean (s.d.)13 6 months.	↔CD4, ↔Viral load	Austin *et al.* 2006
EEUU	Adults	262	Selenium 200µg daily 9 months	↔CD4, ↔Viral load, (+) Se	Hurwitz *et al.* 2007
Tanzania	Adults	1148	Multivitamins Daily for 24 months	↔CD4, ↔Viral load, (+) Body weight	Villamor *et al.* 2008

Legend *Number of studied individuals. **Statistical analysis with significant difference ($p<0.05$). – diminish ↔ no change + increment respect the beginning of intervention.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

In the Multicenter AIDS Cohort Study (MACS), higher dietary intakes of these vitamins, along with vitamin B-6, were associated with a slower progression to AIDS, but only niacin remained significantly associated in the multivariate model. A high intake of B vitamins and use of vitamin B supplements were also associated with improved survival in this cohort.

Progression to AIDS was slower with a higher intake of vitamins C and E among subjects in the SFMHS. In the MACS cohort, subjects with the highest quartile of vitamin C intake had significantly more AIDS-free time than did those with lower intakes, but no significant difference was observed with intakes of vitamin E. Time to death did not vary by intake of either nutrient in this cohort. However, the serum vitamin E concentration in the highest quartile was associated with a 34% decrease in the risk of progression to AIDS compared with the lowest quartile. Nevertheless, the serum vitamin E concentration was not correlated with changes in CD4+ counts over time or with risk of death (*134*).

Studies focused on beta carotene and vitamin A gave disparate results and presently is not considered of benefit in HIV-infected patients (*133*). Selenium and probably iron are the only trace elements that remained as independent predictors of mortality after multivariate adjustment.

Gil *et al.* (2005) observed an increase in TAC, GSH and GPx activity and decrease in SOD activity during the examination of 40 institutionalized HIV individuals at the beginning and the end of an increment in micronutrient intake during seasonal period of three months. During this period the fresh fruit and vegetable are more available and diet offers were increased in correspondence with increase of dietary intake of Vit A, E and C ($p<0.05$ for all). Simultaneous improved antioxidant status and increased micronutrients intake occur in 69 % of

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

HIV patients and it is related with a significant decrease in CD38+/CD8 count, a subset of lymphocyte that correlate with viral load and progression (*63*).

Improvement in GSH redox status and an increase in the antioxidant vitamins without full normalization have been reported by other researcher who evaluated this parameter in 20 HIV + individuals with HAART in 72 weeks follow up (*126*).

Micronutrient supplementation in a few studies during pregnancy has been shown to reduce the risk of low birth weight and preterm birth. These birth outcomes may be risk factors for vertical HIV transmission during delivery or breast-feeding. Use of multivitamin and mineral supplements by low-income pregnant women in the United States is associated with a 41% reduction in the risk of low birth weight and a 34% reduction in the risk of preterm birth. There was no significant effect on the risk of small-for-gestational-age births. However, supplement use was not randomized so there may have been residual confounding of the observed associations despite multivariate adjustment for known confounders such as parity, prepregnancy body mass index, gestational weight gain, smoking, and previous poor birth outcome. The observed beneficial effect of micronutrients is probably mediated through improvement in fetal nutritional status, enhancement of fetal immunity, and decreased risk of infections, which together would decrease risks of intrauterine growth retardation, fetal loss, preterm birth, and low birth weight. However, how the individual vitamins produce these effects is not fully understood. Vitamins may increase placental weight, which will increase the surface area available for maternal-fetal exchange, since placentas of undernourished women are lighter and have fewer villi than those of well-nourished women (*133*).

The HIV infected population spends large sums of money each year on various mineral and natural antioxidants supplements but not clinical trials have rigorously investigated the effects of these supplementations.

Flavonoids as natural products have free radicals-scavenging abilities and, their anti-radicals property is directed towards $^{\bullet}OH$ and $O_2^{\bullet-}$ which are remarkable ROS implicated in the initiation of lipid peroxidation. Their antioxidant function may be synergistically promoted through the activation of GPx dependent on the Se sufficiency (*2*, *67*).

Study assessed with Korean red ginseng (KRG) suggests its long-term immune modulation effects without side effects on HIV-infected patients. This study carried out during 2 years showed increased levels of CD4 ($p<0.01$) in 10 of 12 patients involved in daily supplementation with 5.4 g of KRG. But not redox markers were evaluated and it not associated with antioxidant properties (*130*).

Vimang® is an extract obtained from the stem bark of selected varieties of *Mangifera indica* L. and contains a mixture of components (polyphenols, terpenoids, steroids, fatty acids and microelements) (Centre of Pharmaceutical Chemistry, 1998) (*137*). Vimang® has shown a powerful scavenger activity of hydroxyl radicals ($^{\bullet}OH$) and hypochlorous acid, a significant inhibitory effect on the peroxidation of rat brain phospholipids, and inhibited DNA damage induced by iron/bleomycin or copper-phenantroline systems. Vimang® also inhibits microsomal lipid peroxidation, neuronal death by ischemic reperfusion and provided better protection than Vit. E,C and β-caroteno against TPA-induced oxidative damage in mice. Vimang® has been also tested in a broad set of

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

toxicological tests with satisfactory results, in terms of acute and sub-chronic toxicity, genotoxicity, and irritability demonstrating that it can be classified as a non-toxic product (*138–141*).

Vimang® supplementation during 6 months significantly decreases oxidative damage and contributes to increase of antioxidant capacity in a group of 30 HIV infected patients. Furthermore with this supplementation there was a trend towards a reduction in CD95 with no influencing on CD4 subset count (*137*).

Hurwitz *et al.* (2007) find that dietary supplementation of Se (200 mg) per day to HIV-infected subjects increased blood Se levels, increased T cell count and decreased viral load (*64*). Further, a 5-year randomized, double-blind placebo-controlled clinical trial in Tanzania found a 5% decrease in risk of mortality with each increase of 0.01 μMol Se/L blood from dietary supplementation in HIV-infected pregnant women.

Potential synergistic activity between combination of antiviral therapy and antioxidant supplementation should be explored.

The supplementation with antioxidants, insulin-sensitizing agents, and human growth factor in HIV-1 infected patients with lipodystrophy failed to demonstrate a significant benefit. The growth hormone releasing hormone analogue seem to be the most promising to date but the very small number of randomized and controlled studies leaves the question about their utility still open. Despite that, dietary supplements and antioxidants to reverse fat maldistribution are widespread in HIV-1-infected patients, who take them without medical prescription (*62*, *125*, *142–144*).

A pilot study assessed antioxidant supplementation on mitochondrial function, fat distribution, and lipid and glucose metabolism in HIV-1-infected patients with antiretroviral therapy (ART)-related lipoatrophy. Sixty one ART-treated HIV-1-infected patients with lipoatrophy were randomized to receive either n-acetyl-L-carnitine (n=21), lipoic acid+n-acetylcisteine (LA/NAC) (n=20), or no supplementation (n=20) for 48 weeks. mtDNA content increased in CD4+ T-cells from patients who received n-acetyl-carnitine (+30 copies/cell; p=0.03), without significant difference by the overall comparison of the study groups. Fat body mass and lipid profile did not change significantly in any of the arms. Authors showed that antioxidant supplementation might play a protective role on mitochondrial function, but with only very limited effects on the reversal of lipodistrophic abnormalities in HIV-1-infected patients on long-term HAART (*142*).

Reducing or delaying mitochondrial damage and the OS by using antioxidants might represent, potentially, a therapeutic strategy for ART-related lipodystrophy. Acetyl-L-carnitine and lipoic acid (LA) have been found to be protective nutrients of mitochondria. *In vitro* findings showed that acetyl-L-carnitine facilitates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation and the removal of excess short- and medium-chain fatty acids; it also enhances acetylcholine production and stimulates protein and membrane phospholipid synthesis. Acetyl-L-carnitine has been found to be depleted in patients with HAART-related neurotoxicity and it was proposed as a therapeutic agent for distal symmetrical polyneuropathy in HIV-1-infected patients. LA supplementation decreases oxidative stress *in vivo* by reacting with oxygen species, by protecting membranes through the interaction with vitamin C, and by increasing intracellular content of glutathione and l-cysteine. Acetyl-L-carnitine and LA combination synergistically promote mitochondrial synthesis and adipocyte metabolism *in vitro* through an increased expression of peroxisome proliferator-activated receptors (PPARs) PPARγ and PPARα mRNA, possibly improving insulin sensitivity (*142*).

Additional adverse effects and/or regimen adherence difficulties to HAART have serious consequences such as loss of serum HIV suppression, development of drug-resistant HIV strains, and increased probability of illness progression (*64*).

The OS effects due to phophorylated-NRTI mitochondrial toxicity may amplify some of the pathophysiology and phenotypic events during infection (*64*, *105*, *108*). This aspect result in a new tissue target as treatment is prolonged with increased longevity from AIDS, resistance to NRTI appears, and AIDS become a relatively manageable chronic illness.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

HIV Infection, Redox Regulation, and Physical Exercise

Until the efficacy and safety of pharmacological interventions are rigorously studied in persons with HIV, the cornerstone of management for central fat accumulation should be diet and exercise, as extrapolated from populations not infected with HIV (*145*). For central fat accumulation, the goal should be to decrease intake of saturated fat and excess caloric energy. Aerobic exercise is expected to augment the effects of dietary change because intra-abdominal fat (mesenteric and omental adipose tissue) is metabolically more active than peripheral fat in responding to lipolytic stimuli, such as increases in epinephrine with exercise. In persons without HIV, intensive aerobic exercise can decrease intra-abdominal adipose tissue by 17%-20% (*146*). In addition, aerobic exercise increases peripheral glucose disposal in obese persons, even in the absence of weight loss (*147*), and should be beneficial for subjects with insulin resistance. A recent report suggests that an aerobic exercise program with a moderate-fat, low-glycemic-index, high-fiber diet can reverse aspects of lipodystrophy (*148*).

Exercise training has been used as non pharmacological treatment to promote improvement in anthropometric, cardiorespiratory, muscular and psychological outcomes in HIV infected individuals (*149–151*).

Physical exercise due to higher oxygen update may promote an increase in production of ROS by mitochondria and can therefore influence on the redox state of the cell. Also muscular damage subsequent to exercise can cause inflammation and release superoxide by NADPH from neutrophyls. Physical exercise promotes a transient increase in the production of ROS which conceivable produce an exposure/withdrawal in an environment of redox imbalance has been suggested as a factor responsible for adaptation of exercise-induced antioxidant mechanisms (*152*, *153*).

The training regular responses are assumed as similarly to the HIV negative individuals.

Some investigations have shown significant benefits of aerobic exercise in HIV-infected persons in the areas of cardiorespiratory capacity, immune status, and metabolic activity as well as psychological variables such as a reduction in

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

symptoms of depression and anxiety (*149*, *150*, *154*, *155*). These benefits have been observed in as little as 6 weeks of training from aerobic activity at any intensity if performed at least 2 to 3 sessions per week. However, a comparison across intensities is difficult, as most of the studies did not equalize the dosage of exercise across the intensity range.

Physiological variables measured in aerobic exercise interventions in HIV-infected populations include body composition and high-density lipoprotein (HDL) cholesterol. A 16-week intervention in a group composed of men and women developed a lower intensity aerobic exercise regimen (~40% VO2max), twice a week for 45 minutes. There were significant increases ($p<0.05$) in VO2max and HDL cholesterol, as well as significant decreases ($p<0.05$) in total abdominal adipose tissue, total cholesterol, and triglycerides. Similarly, other group reported a significant increase ($p<0.05$) in VO2max as well as decreases in BMI, waist-to-hip ratio, body density, and body fat after a 12-week moderate-intensity aerobic exercise intervention (~60% of HRmax) performed for 30 minutes a day, 3 days a week.

A study carried out a comparisson between the levels of OS markers and the immunological response profile in HIV-infected and non-HIV subject participating in a single session of aerobic exercise followed by one session of resistance exercise, both at moderate intensity is reported (*156*).

The exercise protocol consisted of a single session of 20 minutes on a cycloergometer followed by a set of six resistance exercises. The activity of glutathione S-transferase (GST) and catalase were measured in plasma samples, total glutathione (TGSH) and thiobarbituric acid reactive substances (TBARS) were measured in erythrocytes. T CD4+ cells, T CD8+, viral load, complete blood count, and white blood count were also assessed. All measurements were performed at three times: baseline, after aerobic exercise, and after resistance exercises. At baseline, the HIV group had lower GST activity than controls, but after the exercise session GST values were similar in both groups. Compared to the control group TGSH was significantly lower ($p<0.05$) in the HIV group at baseline, after aerobic and resistance exercises (*156*). The control group presented higher TBARS values after aerobic exercise compared to the HIV group. The neutrophil count was lower in the HIV group after aerobic and resistance exercises. Similarly, the exercise session did not significantly change T CD4+ count and viral load among HIV subjects, suggesting that exercise was performed at a safe intensity, as previously demonstrated in other study (*150*).

These data indicate that HIV-infected subjects had lower antioxidant activity at rest. Physical exercise stimulated the enzymatic activity similarly in both groups.

The increase of the antioxidant capacity after physical exercise in HIV individuals has been found by several authors as well as higher concentration in the non enzymatic antioxidant such uric acid, ascorbic acid and α tocopherol. This effect produces adaptation and promotes greater tissue resistance to oxidative challenge.

Based on these results physical training (mixed aerobic and strength) may be safely performed by HIV infected individuals. The physical schedule program constitutes a complementary strategy providing induction of antioxidant response which produces a beneficial adaptation capable to mitigate the deletereal effects of OS.

Although the general goal for diet is caloric energy restriction to reduce central fat, the prescribing of specific changes for intake of macronutrients (fats and carbohydrates) should be based on serum lipid levels and glucose intolerance (*151*).

A relatively strong body of literature illustrates the beneficial effects of combined aerobic and resistance exercise training for those infected with HIV. The advantage of combined exercise training is enhanced cardiorespiratory function in a population that is typically deconditioned with the strength and muscle mass gains of resistance training.

Results to date indicate that moderate- to high-intensity aerobic, resistance, and combined aerobic and resistance exercise regimens can be safe and elicit favourable and beneficial changes in the HIV-infected population. These benefits can include changes in body composition, functional capacity, muscular strength, total and HDL cholesterol, cognitive function, depression and anxiety, overall health, and quality of life. Most studies indicate no beneficial effect of exercise training on HIV status, viral load, or immune function (*155*). However, aerobic exercise has shown no negative effect on immunity or disease progression. The effects of resistance or combined exercise programs on immunity have yet to be determined.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Concluding Remarks

Advanced in HIV research allow the scientific community to better understand the molecular and clinic mechanism. Oxidative stress underlying HIV evolution causes a very wide spectrum of genetic, metabolic, and cellular response from diverse tissues overwhelms the organism. This impact contributing to the spectrum of malignancies associated to HIV infection. The OS evaluations will therefore become potential utility factors to follow antiviral combinations effects, as well as the usefulness of antioxidant and alternative therapies. The counteract actions strategy to diminish the impact of oxidative damage may contribute both restoration of immune response and down regulation of HIV replication but also may attenuate the toxic effects of HAART which will impact on quality of live of infected individuals.

References

1. UNAIDS: AIDS Epidemic Update, January 2011. http://www.unaids.org/.
2. Sahnoun, Z.; Jamoussi, K.; Zeghal, K. M. *Therapie* **1997**, *52* (4), 251–270.
3. Block, G.; Dietrich, M.; Norkus, E. P.; Morrow, J. D.; Hudes, M.; Caan, B.; Packer, L. *Am. J. Epidemiol.* **2002**, *156* (3), 274–285.
4. Buhl, R.; Jaffe, H. A.; Holroyd; Well, F. B.; K., J.; Mastrangeli, A.; Saltini, C.; Cantin, A. M.; Crystal, R. G. *Lancet* **1989**, *2*, 1294–1298.
5. Sonnerborg, A.; Carlin, G.; Akerlund, B.; Jarstrand, C. *Scand. J. Infect. Dis.* **1988**, *20*, 287–290.

6. Droge, W. *Physiol. Rev.* **2002**, *82* (1), 47–95.
7. Schreck, R.; Rieber, P.; Baeuerle, P. A. *EMBO* **1991**, *10*, 2247–2258.
8. Seve, M.; Favier, A.; Osman, M.; Hernández, D.; Vaitaitis, G.; Flores, N. C.; McCord, J. M.; Flores, S. C. *Arch. Biochem. Biophys.* **1999**, *361* (2), 165–172.
9. Westendorp, M. O.; Frank, R.; Ochsenbauer, C.; Stricker, K.; Dhein, J.; Walczak, H.; Debatin, K. M.; Krammer, P. H. *Nature* **1995**, *375*, 497–500.
10. Gehri, R.; Hahn, S.; Rothen, M.; Steuerwald, M.; Nuesch, R.; Erb, P. *AIDS* **1996**, *10*, 9–16.
11. Greenspan, H. C.; Aruoma, O. *Chem. Biol. Interact.* **1994**, *143*, 145–148.
12. Kruman, I.; Nath, A.; Mattson, M. *Exp. Neurobiol.* **1998**, *154*, 276–88.
13. Semba, R. D.; Tang, A. M. *Br. J. Nutr.* **1999**, *81*, 181–189.
14. Tang, A. M.; Graham, N. M. H.; Kirby, A. J.; McCall, L. D.; Willet, W. C.; Saah, A. J. *Am. J. Epidemiol.* **1993**, *138*, 937–951.
15. Baum, M. K.; Shor-Posner, G.; Lu, Y.; Rosner, B.; Sauberlich, H. E.; Fletcher, M. A.; Szapocznik, J.; Eisdorfer, C.; Buring, J. E.; Hennekens, C. H. *AIDS* **1995**, *9*, 1051–1056.
16. Baum, M. K.; Shor-Posner, G.; Bonvehi, P.; Cassetti, I.; Lu, Y.; Mantero-Atienza, E.; Beach, R. S.; Sauberlich, H. E. *Ann. NY Acad. Sci.* **1992**, *669*, 165–173.
17. Mahalingam, M.; Pozniac, A.; McManus, T. J.; Vergani, D.; Peakman, M. *Clin. Exp. Immnunol.* **1995**, *102*, 481–486.
18. Kedzierska, K.; Crowee, S. M. *Curr. Med. Chem.* **2002**, *9* (21), 1893–1903.
19. Gorry, P. R.; Ancuta, P. *Curr HIV/AIDS Rep.* **2011**, *8* (1), 45–53.
20. Toma, J.; Whitcomb, J. M.; Petropoulos, C. J.; Huang, W. *AIDS* **2010**, *24* (14), 2181–2186.
21. Oliver, R. T. *Nature* **2000**, *406*, 673.
22. García, F.; Alvarez, M.; Bernal, C.; Chueca, N.; Guillot, V. *Enferm. Infecc. Microbiol. Clin.* **2011** (4), 297–307.
23. Motozono, C.; Mwimanzi, P.; Ueno, T. *Protein Cell* **2010**, *1* (6), 514–519.
24. Froebel, K. S.; Raab, G. M.; D'Alessandro, C.; Armitage, M. P.; MacKenzie, K. M.; Struthers, M.; Whitelaw, J. M.; Yang, S. *Clin. Exp. Immunol.* **2000**, *122* (1), 72–78.
25. Jackson, D. G.; Bellesia, E. I. *J. Immunol.* **1990**, *144*, 2811–2815.
26. Aarhus, R.; Graeff, R. M.; Dickey, D. M.; Walseth, T. F.; Lee, H. C. *J. Biol. Chem.* **1995**, *270*, 30327–30333.
27. Alessio, M.; Roggero, S.; Funaro, A.; De Monte, LBPL; Geuna, M.; Malavasi, F. *J. Immunol.* **1998**, *145*, 878–884.
28. Sloand, E. M.; Young, N. S.; Kumar, P.; Weichold, F. F.; Sato, T.; Maciejewski, J. P. *Blood* **1997**, *89*, 1357–1363.
29. Bohler, T.; Baumler, C.; Herr, I.; Groll, A.; Kurz, M.; Debatin, K. M. *Pediatr. Infect. Dis. J.* **1997**, *16*, 754–759.
30. Aries, S. P.; Schaaf, B.; Muller, C.; Dennin, R. H.; Dalhoff, K. *J. Mol. Med.* **1995**, *73*, 591–593.
31. Fuchs, J.; Schofer, H.; Ochesendorf, F.; Janka, S.; Milbradt, R.; Buhl, R.; Unkelbach, U.; Freisleben, H. J.; Oster, O.; Siems, W.; Grune, T.; Esterbauer, H. *Eur. J. Dermatol.* **1994**, *4*, 148–153.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

32. Israel, N.; Gougerot- Pocidalo, M. A. *CMLS* **1997**, *53*, 864–870.
33. Gil, L.; Martínez, G.; González, I.; Alvarez, A.; Molina, R.; Tarinas, A.; León, O. S.; Pérez, J. *Pharmacol. Res.* **2003**, *47*, 217–224.
34. Aquaro, F.; Scopelliti, F.; Pollicita, M.; Perno; Federico, P. *Future HIV Ther.* **2008**, *2* (4), 327–338.
35. Ma, Q. *Phamacol. Ther.* **2010**, *125*, 376–393.
36. Zhang, H. S.; Sang, W. W.; Ruan, Z.; Wang, Y. O. *Arch. Biochem. Biophys.* **2011**, *505* (2), 266–272.
37. Zwart, L. L.; Meerman, J. H. N.; Commandeur, J. N. M.; Vermeulen, N. P. E. *Free Radical Biol. Med.* **1999**, *26* (1/2), 202–226.
38. Halliwell, B.; Whiteman, M. *Br. J. Pharmacol.* **2004**, *142*, 231–255.
39. Favier, A.; Sappey, C.; Leclerc, P.; Faure, P.; Micoud, M. *Chem.-Biol. Interact.* **1994**, *91*, 165–180.
40. Repetto, M.; Reides, C.; Gómez, M. L.; Costa, M.; Griemberg, G.; Llesuy, S. *Clin. Chim. Acta* **1996**, *255*, 107–117.
41. Look, M. P.; Rockstroh, J. K.; Rao, G. S.; Kreuzer, K. A; Barton, S.; Lemoch, H.; Sudhop, T.; Hoch, J.; Stockinger, K.; Spengler, U.; Sauerbruch, T. *Eur. J. Clin. Nutr.* **1997**, *51*, 266–272.
42. Herzenberg, L.; De Rosa, S. C.; Dubs, J. G.; Roederer, M.; Anderson, M. T.; Ela, A. W.; Deresinski, S. C.; Herzenberg, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1967–1972.
43. Walmsley, Sh. L.; Louise, M. W.; Maureen, L. H.; Jack, P. V.; Peter, G. W. *AIDS* **1997**, *11*, 1689–1697.
44. Allard, J.; Aghdassi, E.; Chau, J.; Salit, I.; Walmsley, Sh. L. *Am. J. Clin. Nutr.* **1998**, *67*, 143–147.
45. Gil, L.; González, I.; Tarinas, A.; Alvarez, A.; Molina, R.; Tápanes, R.; Pérez, J. *Latin Am. J. Pharm.* **2004**, *23* (4), 466–471.
46. Suresh, D. R.; Annam, V.; Pratibha, K.; Prasad, M. *J. Biomed. Sci.* **2009**, *16*, 61.
47. Coaccioli, S.; Crapa, G.; Fanetra, M.; Del Giorno, R.; Lavagna, A.; Standoli, M. L.; Frongillo, R.; Biondi, R.; Puxeddu, A. *Clin. Ter.* **2010**, *161* (1), 55–58.
48. Taylor, W.; Nadimpalli, R. G.; Ramanathan, C. S. *Biol. Trace Element Res.* **1997**, *56*, 63–91.
49. Harthill, M. *Biol. Trace Elem. Res.* **2011**, DOI: 10.1007/s12011-011-8977-1.
50. Kupka, R.; Msamanga, G. I.; Spiegelman, D.; Morris, S.; Mugusi, F.; Hunter, D. J.; Fawzi, W. W. *J. Nutr.* **2004**, *134*, 2556–2560.
51. Gendron, K.; Ferbeyre, G.; Heveker, N.; Brakier-Gingras *Nucleic Acid Res.* **2011**, *39* (3), 902–912.
52. Sacktor, N.; Haughey, N.; Cutler, R.; Tamara, A.; Turchan, J.; Pardo, C.; Vargas, D.; Nath, A. *J. Neuroimmunol.* **2004**, *157* (1−2), 176–184.
53. Price, T. O.; Ercal, N.; Nakaoke, R.; Banks, W. A. *Brain Res.* **2005**, *1045*, 57–63.
54. Price, T. O.; Uras, F.; Banks, W. A.; Ercal, N. *Exp. Neurol.* **2006**, *201*, 193–202.
55. Kotler, D. P.; Tierney, A. R.; Wang, J.; Pierson, R. N. *Am. J. Clin. Nutr.* **1989**, *50*, 444–447.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

56. Abrams, B.; Duncan, D.; Hertz-Picciotto, I. *J. Acquired Immune Defic. Syndr.* **1993**, *6*, 949–958.
57. Suttmann, U.; Ockenga, J.; Selberg, O.; Hoogestraat, L.; Deicher, H.; Muller, M. J. *J. Acquired Immune Defic. Syndr.* **1995**, *8*, 239–246.
58. Castetbon, K.; Kadio, A.; Bondurand, A.; Yao, A. B.; Barouan, C.; Coulibaly, Y.; Anglaret, X.; Msellati, P.; Malvy, D.; Dabis, F. *Eur. J. Clin. Nutr.* **1997**, *51*, 81–86.
59. Skurnick, J. H.; Bogden, J. D.; Baker, H.; Kemp, F. W.; Sheffet, A.; Quattrone, G.; Louria, D. B. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **1996**, *12*, 75–83.
60. Irlam, J. H.; Visser, M. M. E.; Rollins, N. N.; Siegfried, N. *Cochrane Database of Systematic Reviews*; 2010, 12. Art. No.: CD003650, DOI: 10.1002/14651858.CD003650.pub3.
61. Gil, L.; Ferreira, R. *Antioxidants and Quality of Life*; September 2004. http://www.antioxidantes.com.ar/12/FrArt242.htm.
62. Austin, J.; Singhal, N.; Voigt, R.; Smaill, F.; Gill, M. J.; Walmsley, S, Salit, I.; Gilmour, J.; Schlech, W. F., III; Choudhri, S.; Rachlis, A.; Cohen, J.; Trottier, S.; Toma, E.; Phillips, P.; Ford, P. M.; Woods, R.; Singer, J.; Zarowny, D. P.; Cameron, D. W. CTN 091/CRIT Cartenoids Study Group. *Eur. J. Clin. Nutr.* **2006**, *60* (11), 1266–1276.
63. Gil, L.; Lewis, L.; Martínez, G.; Tarinas, A.; González, I.; Alvarez, A.; Tápanes, R.; Giuliani, A.; León, O. S.; Pérez, J. *Int. J. Vitam. Nutr. Res.* **2005**, *75* (1), 19–27.
64. Hurwitz, B. E.; Klaus, J. R.; Llabre, M. M.; González, A.; Lawrence, P. J.; Maher, K. J.; Greeson, J. M.; Baum, M. K.; Shor-Posner, G.; Skyler, J. S.; Schneiderman, N. *Arch. Int. Med.* **2007**, *167* (2), 148–154.
65. Bilbis, L. S.; Idowu, D. B.; Saidu, Y.; Lawal, M.; Njoku, C. H. *Ann. Africa Med.* **2010**, *9* (4), 235–239.
66. World Health Organisation. *WHO Technical Consultation on Nutrient Requirements for People Living with HIV/AIDS*; World Health Organisation: Geneva, 2003.
67. Pérez, G.; Sánchez, G. M. *Acta Farm. Bonaerense* **2001**, *20* (4), 297–306.
68. Nagata, H.; Takekoshi, T.; Takagi, T.; Honma, T.; Watanabe, K. *Tokai J. Exp. Clin. Med.* **1999**, *24* (1), 1–11.
69. De Clercq, E. *Rev. Med. Virol.* **2009**, *19*, 287–299.
70. *Guidelines for the Use of Antiretroviral Agents in HIV-Infected Adults and Adolescents*; Department of Health and Human Services (DHHS), 2008.
71. Lewis, W. *Antiviral Res.* **2003**, *58*, 189–197.
72. Brinkman, K.; Smeitink, J. A.; Romijin, J. A.; Reiss, P. *Lancet* **1999**, *354* (9184), 1112–1115.
73. Kakuda, TN. *Clin. Ther.* **2000**, *22* (6), 685–708.
74. Lewis, W.; Copeland, W. C.; Day, B. *Lab. Invest.* **2001**, *81* (6), 777–790.
75. Moyle, G. *Clin. Ther.* **2000**, *22*, 911–936.
76. Casademont, J.; Niro, O.; Cardellach, F. *N. Engl. J. Med.* **2002**, *347* (3), 216–218.
77. Feng, J. Y.; Johnson, K. A.; Anderson, K. S. *J. Biol. Chem.* **2001**, *276* (26), 23832–23837.

78. Schwartz, R. M.; Dayhoff, M. O. *Science* **1978**, *199*, 395–403.
79. Gary, M. W.; Doolittle, WF. *Microbiol. Rev.* **1982**, *46*, 1–42.
80. Kornburg, H. L. *Bioassays* **1987**, *7*, 236–238.
81. DiMauro, S.; Schon, E. A. *Am. J. Med. Genet.* **2001**, *106*, 18–26.
82. Staniek, K.; Gille, L.; Kozlov, A. V.; Nohl, H. *Free Radical Res.* **2002**, *36* (4), 381–387.
83. Johnson, A. A.; Ray, A. S.; Hanes, J.; Suo, Z.; Colacino, J. M.; Anderson, K. S.; Johnson, K. A. *J. Biol. Chem.* **2001**, *276* (44), 40847–40857.
84. Lim, S. E.; Copeland, W. C. *J. Biol. Chem.* **2001**, *276* (26), 23616–23623.
85. Honkoop, P.; Scholte, H. R.; de Man, R. A.; Schalm, S. W. *Drug Saf.* **1997**, *17* (1), 1–7.
86. Lewis, W. *Am. J. Physiol. Heart Circ. Physiol.* **2003**, *284* (1), H1–H9.
87. Yakes, F. M.; Van Houten, B. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (2), 514–519.
88. Walker, U. A. *J. HIV Ther.* **2001**, *6* (1), 17–21.
89. Wanke, C. A. *AIDS* **1999**, *13* (11), 1287–1293.
90. Safrin, S.; Grunfeld, C. *AIDS* **1999**, *13* (18), 2493–2505.
91. Reaven, G. M. *J. Intern. Med. Suppl.* **1994**, *736*, 13–22.
92. Mynarcik, D. C.; McNurlan, M. A.; Steigbigel, R. T.; Fuhrer, J.; Gelato, M. C. *J. Acquired Immune Defic. Syndr.* **2000**, *25* (4), 312–321.
93. Szabo, C.; Day, B. J.; Salzman, A. L. *FEBS Lett.* **1996**, *381* (1-2), 82–86.
94. Estrada, V.; Serrano-Rios, M.; Martinez Larrad, M. T.; Villar, N. G.; González López, A.; Tellez, M. J.; Fernández, C. *J. Acquired Immune Defic. Syndr.* **2002**, *29* (1), 32–40.
95. Hadigan, C.; Meigs, J. B.; Corcoran, C.; Rietschel, P.; Piecuch, S.; Basgoz, N.; Davis, B.; Sax, P.; Stanley, T.; Wilson, P. W.; D'Agostino, R. B.; Grinspoon, S. *Clin. Infect. Dis.* **2001**, *32* (1), 130–139.
96. Brinkman, K. *J. HIV Ther.* **2001**, *6* (1), 13–16.
97. Brinkman, K.; Vrouenraets, S.; Kauffmann, R.; Weigel, H.; Frissen, J. *AIDS* **2000**, *14* (17), 2801–2802.
98. Ter Hofstede, H. J.; De Marie, S.; Foudraine, N.; Danner, S.; Brinkman, K. In *Proceedings of the 7th Conference of Retroviruses and Opportunistic Infections*, San Francisco, CA, 2000.
99. Lonergan , J. T.; Havlir, D.; Behling, C.; Pfander, H.; Hassanein; T.; Mathews, W. C. In *Proceedings of the 7th Conference on Retroviruses and Opportunistic Infections*, San Francisco CA, January 30−February 2, 2000.
100. Gerschenson, M.; Nguyen, V. T.; St Claire, M. C.; Harbaugh, S. W.; Harbaugh, J. W.; Proia, L. A.; Poirier, M. C. *J. Hum. Virol.* **2000**, *4* (60), 335–342.
101. Lewis, W.; Grupp, I. L.; Grupp, G.; Hoit, B.; Morris, R.; Samarel, A. M.; Bruggeman, L.; Klotman, P. *Lab. Invest.* **2000**, *80* (2), 187–197.
102. Prakash, O.; Teng, S.; Ali, M.; Zhu, X.; Coleman, R.; Dabdoub, R. A.; Chambers, R.; Yee, A. T.; Flores, S. C.; Joshi, B. H. *Arch. Biochem. Biophys.* **1997**, *343* (2), 173–180.
103. Moore, R.; Keruly, J.; Chaisson, R. In *Proceedings of the 7th Conference on Retroviruses and Opportunistic Infections*, San Francisco, CA, 2000.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

104. Saint-Marc, T.; Partisani, M.; Poizot-Martin, I.; Bruno, F.; Rouviere, O.; Lang, J. M.; Gastaut, J. A.; Touraine, J. L. *AIDS* **1999**, *13* (13), 1659–1667.
105. White, A. J. *Sex. Transm. Infect.* **2001**, *77* (3), 158–173.
106. Sattler, F. *Clin. Infect. Dis.* **2003**, *36* (suppl 2), s84–s90.
107. Vassimon, H. S.; Deminice, R.; Machado, A. A.; Monteiro, J. P.; Jordão, A. *Curr. HIV Res.* **2010**, *8* (5), 364–369.
108. Carr, A.; Samaras, K.; Thorisdottir, A.; Kaufmann, G. R.; Chisholm, D. J.; Cooper, D. A. *Lancet* **1999**, *353*, 2093–2099.
109. McComsey, G. A.; Paulsen, D. M.; Lonergan, J. T.; Hessenthaler, S. M.; Hoppel, C. L.; Williams, V. C.; Fisher, R. L.; Cherry, C. L.; White-owen, C.; Thompson, K. A.; Ross, S. T.; Hernández, J. E.; Ross, L. L. *AIDS* **2005**, *10* (1), 15–23.
110. Dalakas, M. C.; Semino-Mora, C.; Leon-Monzon, M. *Lab. Invest.* **2001**, *81* (11), 1537–1544.
111. Dalakas, M. C. *J. Peripher. Nerv. Syst.* **2001**, *6* (1), 14–20.
112. Cote, H. C.; Brumme, Z. L.; Craib, K. J.; Alexander, C. S.; Wynhoven, B.; Ting, L.; Wong, H.; Harris, M.; Harrigan, P. R.; O'Shaughnessy, M. V.; Montaner, J. S. *N. Engl. J. Med.* **2002**, *346* (11), 811–820.
113. Lai, Y.; Tse, C. M.; Unadkat, J. D. *J. Biol. Chem.* **2004**, *279* (6), 4490–4497.
114. Hulgan, T.; Morrow, J.; D'Aquila, R.; Raffanti, S.; Morgan, M.; Rebeiro, P.; Haas, D. W. *Clin. Infect. Dis.* **2003**, *37*, 1711–1717.
115. Aukrust, P.; Muller, F.; Svardal, A.; Ueland, T.; Berge, R. K.; Froland, S. S. *J. Infect. Dis.* **2003**, *188*, 232–238.
116. Hurwitz, B. E.; Klimas, N. G.; Llabre, M. M.; Maher, K. J.; Skyler, J. S.; Bilsker, M. S.; McPherson-Baker, S.; Lawrence, P. J.; Laperriere, A. R.; Greeson, J. M.; Klaus, J. R.; Lawrence, R.; Schneiderman, N. *Cardiovasc. Toxicol.* **2004**, *4* (3), 303–316.
117. Ngondi, J. L.; Oben, J.; Forkah, D. M.; Etame, L. H.; Mbanya, D. *AIDS Res. Ther.* **2006**, *3*, 19.
118. Hulgan, T.; Hughes, M.; Sun, X.; Smeaton, L. M.; Terry, E.; Robbins, G. K.; Shafer, R. W.; Clifford, D. B.; McComsey, G. A.; Canter, J. A.; Morrow, J. D.; Haas, D. W. *J. Acquired Immune Defic. Syndr.* **2006**, *42* (4), 450–454.
119. Masia, M.; Padilla, S.; Bernal, E.; Almenar, M. V.; Molina, J.; Hernández, I.; Gutierrez, F. *Clin. Ther.* **2007**, *29* (7), 1448–1455.
120. Mandas, A.; Iorio, E. L.; Congiu, M. G.; Balestrieri, C.; Mereu, A.; Cau, D.; Curreli, N. *J. Biomed. Biotechnol.* 2009, Epub. 749575.
121. Wanchu, A.; Rana, S. V.; Pallikkuth, S.; Sachdeva, R. K. *AIDS Res. Hum. Retrovirol.* **2009**, *25* (12), 1307–1311.
122. Redhage, L. A.; Shintani, A.; Haas, D. W.; Emeagwali, N.; Markovic, M.; Oboho, I.; Mwenya, C.; Erdem, H.; Acosta, E.; Morro, J. D.; Hulgan, T. *HIV Clin. Trials* **2009**, *10* (3), 181–192.
123. Gil, L.; Tarinas, A.; Hernández, D.; Vega, B.; Pérez, D.; Tápanes, R. D.; Capó, V.; Pérez, J. *Biomed. Aging Pathol.* **2011**, DOI: 10.1016/j.biomag.2010.09.009.
124. Caron-Debarle, M.; Boccara, F.; Lagathu, C.; Antoine, B.; Cervera, P.; Bastard, J. P.; Vigouroux, C.; Capeau, J. *Curr. Pharm. Des.* **2010**, *16* (30), 3352–3360.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

125. Milazzo, L.; Menzaghi, B.; Caramma, I.; Nasi, M.; Sangaletti, O.; Cesari, M.; Poma, B. Z.; Cossarizza, A.; Antinori, S.; Galli, M. O. *AIDS Res. Hum. Retroirol.* **2010**, *26* (11), 1207–1124.

126. Villamor, E.; Mugusi, F.; Urassa, W.; Bosch, R. J.; Saathoff, E.; Matsumoto, K.; Meydani, S. N.; Fawzi, W. W. *J. Infect. Dis.* **2008**, *197* (11), 1499–1505.

127. Lagathu, C.; Eustace, B.; Prot, M.; Frantz, D.; Gu, Y.; Bastard, J. P.; Maachi, M.; Azoulay, S.; Briggs, M.; Caron, M.; Capeau, J. *Antivirol. Ther.* **2007**, *12* (4), 489–500.

128. Coutsoudis, A.; Bobat, R. A.; Coovadia, H. M.; Kuhn, L.; Tsai, W. Y.; Stein, Z. A. *Am. J. Public Health* **1995**, *85*, 1076–1081.

129. Coodley, G. O.; Coodley, M. K.; Lusk, R.; Green, T. R.; Bakke, A. C.; Wilson, D.; Wachenheim, D.; Sexton, G.; Salveon, C. *AIDS* **1996**, *10*, 967–973.

130. Cho, Y. K.; H. J. Lee, W. I. Oh, Y. K. Kim. *Abstr. Gen. Meet. Am. Soc. Microbiol.* **1997**, *97*, 247 (abstract Nº E44).

131. Look, M. P.; Rockstroh, J. K.; Rao, G. S.; Barton, S.; Lemonch, H.; Kaiser, R.; Kupfer, B.; Sudhop, T.; Spengler, U.; Sauerbruch, T. *Eur. J. Clin. Invest.* **1998**, *28*, 389–397.

132. Hussey, G., Hughes, J.; Potgieter, S.; Kessow, G.; Burgess, J.; Beatty, D.; Keraan, M.; Carlesle, E. In Abstracts of the XVII International Vitamin A Consultative Group Meeting, Guatemala City, 1996; International Life Science Institute: Washington, DC; p 6.

133. Fawsi, W. W.; Msamanga, G. I.; Spiegelman, D.; Urassa, E. J. N.; McGrath, N.; Mwakagile, D.; Antelman, G.; Mbise, R.; Herrera, G.; Kapiga, S.; Willett, W.; Hunter, D. J. IV Tanzania Vitamin and HIV Infections Trial Team *Lancet* **1998**, *351*, 1477−1482.

134. Allard, J. P.; Aghdassi, E.; Chau, J.; Tam, C.; Kovacs, C.; Salit, I. E.; Walmsley, Sh. L. *AIDS* **1998**, *12*, 1653–1659.

135. Mocchegiani, E.; Veccia, S.; Ancarani, F.; Scalise, G.; Fabris, N. *Int. J. Immunopharmacol.* **1995**, *17*, 719–727.

136. De Rosa, S. C.; Zaretsky, M. D.; Dubs, J. G.; Roederer, M.; Anderson, M.; Green, A.; Mitra, D.; Watanabe, N.; Nakamura, H.; Tjioe, I.; Deresinski, S. C.; Moore, W. A.; Ela, S. W.; Parks, D.; Herzenberg, L. A.; Herzenberg, L. A. *Eur J Clin. Invest.* **2000**, *30*, 915–929.

137. Gil, L.; Martínez, G.; González, I.; Tarinas, A.; Alvarez, A.; Molina, R.; Robaina, M.; Tápanes, R.; Pérez, J.; Guevara, M.; Nuñez, A.; León, O. S. *Free Radical Res.* **2002**, *36* (Supplement 1), 107–109.

138. Sánchez, G. M.; Delgado, R.; Pérez, G. D.; Garrido, G.; Núnez-Sellés, A. J.; León, O. S. *Phytother. Res.* **2000**, *15*, 1–4.

139. Sánchez, G. M.; Giuliani, A.; León, O. S.; Pérez-Davison, G. D.; Núñez-Sellés, A. J. *Phytother Res* **2001**, *15*, 581–585.

140. Sánchez, G. M.; Candelario-Jalil, E.; Giuliani, A.; León, O. S.; Sam, S. R; Delgado, R.; Núnez-Sellés, A. J. *Free Radical Res.* **2001**, *35*, 465–473.

141. Sánchez, G. M.; Re, L.; Giuliani, A.; Núnez-Sellés, A. J.; Pérez-Davison, G.; León, O. S. *Pharmacol. Res.* **2000**, *42*, 555–573.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

142. Jaruga, P.; Jaruga, B.; Gackowski, D.; Olczak, A.; Halota, W.; Pawlowska, M.; Olinski, R *Free Radical Biol. Med.* **2002**, *32* (5), 414–420.
143. Drain, P. K.; Kupka, R.; Mugusi, F.; Fawzi, W. W. *Am. J. Clin. Nutr.* **2007**, *85*, 333–345.
144. Day, B. J.; Lewis, W. *Cardiovasc. Toxicol.* **2004**, *4* (3), 207–216.
145. Ross, R.; Dagnone, D.; Jones, P. J.; Smith, H.; Paddags, A.; Hudson, R.; Janssen, I. *Ann. Intern. Med.* **2000**, *133* (2), 92–103.
146. Schwartz, R. S.; Shuman, W. P.; Larson, V.; Cain, K. C.; Fellingham, G. W.; Beard, J. C.; Kahn, S. E.; Stratton, J. R.; Cerqueira, M. D.; Abrass, I. B. *Metabolism* **1991**, *40* (5), 545–551.
147. Krotkiewski, M.; Bjorntorp, P. *Int. J. Obes.* **1986**, *10*, 331–341.
148. Lamarche, B.; Despres, J. P.; Pouliot, M. C.; Moorjani, S.; Lupien, P. J.; Theriault, G.; Tremblay, A.; Nadeau, A.; Bouchard, C. I *Metabolism* **1992**, *41* (11), 1249–1256.
149. Roubenoff, R.; Schmitz, H.; Bairos, L.; Layne, J.; Potts, E.; Cloutier, G. J.; Denry, F. *Clin. Infect. Dis.* **2002**, *34* (3), 390–393.
150. Roubenoff, R.; Weiss, L.; McDermott, A.; Heflin, T.; Cloutier, G. J.; Wood, M.; Gorbach, S. *AIDS* **1999**, *13* (11), 1373–1375.
151. Hadigan, C.; Jeste, S.; Anderson, E. J.; Tsay, R.; Cyr, H.; Grinspoon, S. *Clin. Infect. Dis.* **2001**, *33* (5), 710–717.
152. Gómez-Cabrera, M. C.; Domenech, E.; Vina, J. *Free Radical Biol. Med.* **2008**, *44* (2), 126–131.
153. Radak, Z.; Chung, H. Y.; Goto, S. *Free Radical Biol. Med.* **2008**, *44* (2), 153–159.
154. Deresz, LF.; Ramos, A; Manfroi, W. C; Gaya, A.; Sprinz, E.; de Oliveira, A. R; Dall'Ago, P. *Rev. Bras. Med. Esporte* **2007**, *13* (4), 249e–252e.
155. Hand, G. A.; Lyerly, W.; Jagger, J. R.; Dudgeon, W. D. *Am. J. Lifestyles Med.* **2009**, *3* (6), 489–499.
156. Deresz, L. F.; Sprinz, E.; Kramer, A. S.; Cunha, G.; de Oliveira, A. R.; Sporleder, H.; de Freitas, D. R.; Lazzarotto, A. R.; Dall'Ago, P. *AIDS Care* **2010**, *22* (11), 1410–1417.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch002

Chapter 3

Thermodynamics of Free Radical Reactions and the Redox Environment of a Cell

Klaudia Jomova[a] and Marian Valko*,[a,b]

[a]Department of Chemistry, Faculty of Natural Sciences,
Constantine The Philosopher University, SK-949 74 Nitra, Slovakia
[b]Faculty of Chemical and Food Technology, Slovak Technical University,
SK-812 37 Bratislava, Slovakia
***E-mail: marian.valko@stuba.sk. Fax: +421-2-524 93 198.**

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

This contribution reports on the thermodynamics of free radical reactions and the maintenance of cell redox homeostasis. Attention is focused on the definition of the half-cell reduction potential and the reducing capacity of the antioxidant couples, the cellular defence system substantiated by the thioredoxin, NADP(H), ascorbic acid, and thiol-containing molecules including glutathione (GSH), cysteine and protein thiols. Triggering of the signalling processes in the cell require the action of the electrochemical potential gradients of the corresponding redox pairs. Experimental quantification of electrochemical potential gradients of the corresponding redox pairs is briefly discussed.

Introduction

Maintenance of a stable redox homeostasis plays a key role in the proper functioning of cellular processes (*1*). Oxidative stress results from altered redox homeostasis and has been linked with the development of many diseases including, cardiovascular disease, diabetes, neurodegenerative disorders, cancer and has also been implicated as an important factor in cell growth regulation and ageing (*2*). Organisms have evolved a complex defence system based on mechanisms comprised of non-enzymatic (low-molecular weight) and enzymatic antioxidants.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

The cellular defence system includes redox buffers such as the thioredoxin, NAD(P)H, ascorbic acid, and thiol-containing molecules including glutathione (GSH), cysteine and protein thiols (*3*). The reducing cellular environment creates the electrochemical gradient necessary for electron transfer in oxidation-reduction reactions occurring in biological systems. The aim of this contribution is to give a brief overview of the thermodynamics of free radical reactions and the basic principles and importance of the maintenance of the redox environment in the cell.

Thermodynamics of Free Radical Reactions

A reductant, or reducing agent, is a substance that loses electrons and causes another species to be reduced. Conversely, an oxidant, or oxidising agent, is a substance that gains electrons and causes another species to be oxidised (*4*). Oxidation (oxidation reaction) is a process in which a substance loses electrons and reduction (reduction reaction) is a process in which a substance gains electrons (*5*). Oxidation and reduction reactions are called redox reactions. Redox reactions represent one of the most common and most important chemical reactions occurring in biological systems. In the biochemistry of free radical reactions we use more frequently the terms antioxidant and pro-oxidant, than the terms reductant and oxidant, respectively (*6*). The thermodynamic principles represent the basis for the prediction of the direction of chemical reactions and thermodynamic quantities can conveniently be used to predict a hierarchy for free radical reactions. Free radicals are species covering a wide range of properties, ranging from those capable of strong oxidation to those capable of strong reduction. One of the most important thermodynamic quantities suitable for the characterization of the course of free radical reactions is the half-cell reduction potential. For example, the one-electron reduction of a compound "A" is related to the half-cell reduction potential of the couple (*7*):

$$\mathrm{A(Ox)} + e^{-} \rightarrow \mathrm{A}^{\bullet-}\mathrm{(Red)} \qquad E[\mathrm{A/A}^{\bullet-}] \qquad (1)$$

The half-cell reduction potentials for many substances can be determined by a variety of electrochemical techniques (*8*). Table 1 summarizes the values of half-field reduction potentials of various species. They are listed from highly oxidising (at the top of Table 1) to highly reducing (at the bottom of Table 1). Oxidised species can accept electrons (hydrogens) from any reduced species occurring below it in Table 1, or, conversely, reduced species are able to donate electrons (hydrogens) to any oxidised species above it in Table 1.

The life time of free radicals is usually very short and therefore it is difficult to maintain the thermodynamically reversible equilibrium at the surface of the electrode in order to determine the exact values of half-cell potentials (*10*). Therefore, a reliable estimate of half-cell potentials requires application of fast kinetic methods (*11*). Table 1 summarises half-cell reduction potentials of selected couples.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

Table 1. Half-cell reduction potentials of selected couples (pH = 7)

Redox couples	*E^0/mV (25°C)*
${}^{\bullet}OH, H^+/H_2O$	+2310
${}^{\bullet}OOH, H^+/H_2O_2$	+1060
${}^{\bullet}OOR, H^+/ROOH$	+770-1440
$O_2{}^{\bullet -}, 2H^+/H_2O_2$	+940
α-TO$^{\bullet}$, H$^+$(Vit. E radical)/ α-TOH (Vit. E)	+500
$H_2O_2, H^+/H_2O, {}^{\bullet}OH$	+320
Asc$^{\bullet -}$,H$^+$ (Ascorbyl rad.)/AscH$^-$ (Ascorbate)	+282
GSSG/2GSH	248

Redox reactions can generally be described by the set of equations:

Redox couple 1 $\quad Red_1 \leftrightarrow Ox_1 + e^-$ (2)

Redox couple 2 $\quad Ox_2 + e^- \leftrightarrow Red_2$ (3)

Overall Redox reaction $\quad Red_1 + Ox_2 \leftrightarrow Ox_1 + Red_2$ (4)

An example of a simple type of redox reaction is:

$$Cu^+ + Fe^{3+} \leftrightarrow Fe^{2+} + Cu^{2+} \tag{5}$$

This reaction consists of two half reactions:

$$Fe^{3+} + e^- \leftrightarrow Fe^{2+} \tag{6}$$

$$Cu^+ \leftrightarrow Cu^{2+} + e^- \tag{7}$$

In this case ferric ions are reduced from the +3 to the +2 oxidation state and cupric ions are oxidised from +1 to +2 oxidation state. In oxidations and reductions occurring in biological systems the electrons are often carried by protons and therefore reactions are pH-dependent, since the concentration of H$^+$ can itself change the half-cell potentials of the redox couples (*12*).

The reaction Gibbs energy change of any reaction also involving a redox reaction is given by the following equations (*12*):

$$\mathrm{D}_r G = \mathrm{D}_r G^{\mathrm{Q}} + RT \ln \frac{[\text{product of activities of all products}]}{[\text{product of activities of all reactants}]} \tag{8}$$

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

where ΔrG^{θ} is the standard reaction Gibbs energy, R is the gas constant (8.314 $Jmol^{-1}\ K^{-1}$), T is the thermodynamic temperature (K). Since $\Delta_r G = -zFE$ we may write:

$$E = E^{Q} - \frac{RT}{zF} \ln \frac{[\text{product of activities of all products}]}{[\text{product of activities of all reactants}]} \tag{9}$$

This equation is called the Nernst equation and provides the electrical potential which can be obtained from an oxidation-reduction reaction. E^{θ} is the standard reduction potential defined as the potential of a couple at unit activity (ln1 = 0). F is the Faraday constant = 96494 C/mol, and z is the number of electrons involved in the process. At equilibrium $\Delta_r G = 0$, thus eqn. 8 simplifies to:

$$\Delta_r G^{\Theta} = -zFE^{\Theta} = -RT \ln K \tag{10}$$

where K is the equilibrium constant of the reaction. For a general chemical reaction (for example: aA + bB ↔ cC + dD), the equilibrium constant is defined by the equation: $K = (a_A{}^a . a_B{}^b)/(a_C{}^c . a_D{}^d)$ where $a_i{}^j$ is the dimensionless quantity called activity, where subscript i refers to chemical species and superscript j refers to stoichiometric coefficients a, b, c and d. They show up as powers of the corresponding reactants and products.

For a spontaneous process, the change in the Gibbs free energy must be negative ($\Delta_r G < 0$), thus from the equation (10) it follows that E must be positive ($E > 0$) (*13*).

For any two redox pairs the overall redox potential is called the electromotive force (ΔE) and is calculated according to the following equation:

$$\mathrm{D}E = E_2(\text{acceptor of electrons}) - E_1(\text{donor of electrons}) \tag{11}$$

The Nernst equation (9) using temperature at t = 25°C (298.15 K) and 2.303 as the conversion factor for the decadic logarithm can be rewritten

$$E = E^{\Theta} - \frac{0.0591}{z} \log K \qquad (\mathrm{V}) \tag{12}$$

Thus from the eqn. (12) one may determine the electrochemical potential between two redox couples.

As an example of these equations we refer here to a very important reaction which takes place in biological systems, namely the regeneration of vitamin E by vitamin C (*14*). Based on the half-cell reduction potential values of the α-tocopheryl radical (α-T-O$^{\bullet}$)/α-tocopherol (α-T-OH, vitamin E) couple and an ascorbate radical (Asc$^{\bullet-}$)/ascorbate monoanion (AscH^{-}, vitamin C) couple it is clear that the ascorbate monoanion can react with the tocopheryl radical to regenerate vitamin E (*15*):

$$\mathrm{AscH^{-}} + \alpha\text{-T-O}^{\bullet} \rightarrow \mathrm{Asc}^{\bullet-} + \alpha\text{-T-OH} \tag{13}$$

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

ΔE is defined as the difference in the half-cell reduction potentials of the two couples:

$$\Delta E \;=\; E_2(\alpha\text{-T-OH}/\alpha\text{-T-O}^\bullet) \;-\; E_1(\text{AscH}^-/\,\text{Asc}^{\bullet-}) \qquad (14)$$

where E_2 is the reduction potential for the half-cell reaction of the species that is reduced (α-T-OH/α-T-O$^\bullet$) and E_1 is the reduction potential for the half-cell reaction of the species that is oxidized (AscH$^-$/Asc$^{\bullet-}$). Thus the electromotive force ΔE for this redox couple ($\Delta E = E_2 - E_1 = 0.5 - 0.282 = 0.218$ V; see Table 1) is positive and consequently the position of the equilibrium can be calculated. As the reduction potential of the E_1 couple is more negative than that of the E_2 couple, ΔE becomes a positive number and ΔG is negative. Hence the equilibrium constant K is positive and so the equilibrium goes to the right-hand side. Thus vitamin C can react with the vitamin E radical and regenerate it.

When discussing reactions occurring in living systems, the thermodynamic principles are not the only ones which should be considered. The issue of kinetics of the process also play an important role, because any reaction that is thermodynamically possible may not be kinetically feasible; in other words the reaction is too slow to be biologically important (*16*).

Since biological systems are not in equilibrium (equilibrium = death), the conditions of reversibility cannot be fulfilled and therefore the redox potential cannot be determined according to its theoretical definition. More suitable is the term redox state which is applied not only to describe the state of a particular redox couple, but also, in the broader sense, to describe the redox environment of a cell (*6*).

Many redox reactions occurring in living systems are represented by two-electron processes, avoiding thus the formation of free radicals (*17*). Similarly to one-electron reactions described above, there is a thermodynamic hierarchy for the two-electron redox reactions (see Table 2). There are two important redox couples (see below) significantly influencing the cellular redox environment. These involve the glutathione system (GSSG/GSH) and the thioredoxin system (TRX-SS/TRX-(SH)2) (*17*). A major source of electrons for reductive biosynthesis including the glutathione and thioredoxin systems is the nicotinamide adenine dinucleotide system (NAD$^+$/NADH) (*18*). The NAD(P)$^+$/NAD(P)H system is described by the reaction

$$\text{NAD(P)}^+ \;+\; 2\text{e}^- \;+\; 2\text{H}^+ \;\rightarrow\; \text{NAD(P)H} \;+\; \text{H}^+ \qquad (15)$$

This reaction can be described by two steps

$$\text{NAD(P)}^+ \;+\; \text{e}^- \;\rightarrow\; \text{NAD(P)}^\bullet \qquad \text{E}^\Theta \;=\; -913\text{ mV} \qquad (16)$$

$$\text{NAD(P)}^\bullet \;+\; \text{e}^- \;+\; 2\text{H}^+ \;\rightarrow\; \text{NAD(P)H} \;+\; \text{H}^+ \qquad \text{E}^\Theta \;=\; +282\text{ mV} \qquad (17)$$

and overall reaction

$$\text{NAD(P)}^+ + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{NAD(P)H} + \text{H}^+ \qquad \text{E}^\Theta = (-913 + 282)/2 = -316\text{ mV} \qquad (18)$$

Table 2. Two-electron reduction potentials of selected couples at pH = 7

Redox couples	*E^0/mV (25°C)*
Xanthine/hypoxanthine, H^+	-371
Uric acid/xanthine, H^+	-360
NAD^+, H^+/NADH	-316
$NADP^+$, H^+/NADPH	-315
GSSG, $2H^+$/2GSH	-240
TRX-SS, $2H^+$/TRX-(SH_2)	-270 to -124 (mean value -240)
GRX-SS, $2H^+$/GRX-(SH_2)	-218
FAD, $2H^+$/$FADH_2$	-219
Pyruvate, $2H^+$/Lactate	-183
Dehydroascorbate, H^+/Ascorbate	+54
Ubiquinone (CoQ), $2H^+$/ubihydroquinone ($CoQH_2$)	+84
O_2, $2H^+$/H_2O_2	+300
H_2O_2, $2H^+$/$2H_2O$	+1320

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

The value of the overall reaction (-316 mV) in which two-electrons are exchanged, can be regarded as the average of the two one-electron reactions.

The half cell reduction potential described by the Nernst equation for the overall reaction ($NAD(P)^+$, H^+/NAD(P)H couple) can be expressed as

$$E = -316 - \frac{59.1}{2} \log \frac{[NAD(P)H]}{[NAD(P)^+]} \quad \text{mV} \ \ (25^{\circ}C, pH = 7) \qquad (19)$$

Assuming the concentrations of NAD(P)H and NAD(P) to be 80μM and 0.8μM, respectively, the equation can be rewritten as (*19*)

$$E = -316 - \frac{59.1}{2} \log \frac{[80]}{[0.8]} \ = \ -375 \text{ mV} \qquad (20)$$

The value of *E* is very negative and is in agreement with the idea that the $NAD(P)^+$/NAD(P)H system is a key player in maintaining the reducing cell environment.

Now we describe the glutathione couple. The half reaction for the GSSG/ 2GSH couple is

$$\text{GSSG} + 2\text{H}^+ \ + \ 2\text{e}^- \ \rightarrow \ 2\ \text{GSH} \qquad (21)$$

and the Nernst equation is given by

$$E = -240 - \frac{59.1}{2} \log \frac{[GSH]^2}{[GSSG]} \quad \text{mV} \;\; (25^{\circ}C, pH = 7) \tag{22}$$

The ratio of reduced to oxidised glutathione is approximately 100:1, thus assuming the concentrations of reduced glutathione and oxidised glutathione are 5 mM and 50 μM, respectively, the Nernst equation is (*20*)

$$E = -240 - \frac{59.1}{2} \log \frac{[5x10^{-3}]^2}{[5x10^{-5}]} \quad \approx \; -300 \text{ mV} \tag{23}$$

The thioredoxin are a family of di-cysteine proteins participating in the electron transfer reactions. The half-reaction for this redox pair can be expressed according to the reaction (see Figure 1) (*21*)

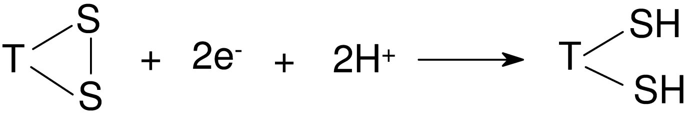


Figure 1. Formation of TRX-(SH)$_2$ from TRX-SS.

In this reaction one molecule of TRX-SS forms one molecule of TRX-(SH)$_2$ and the Nernst equation can be expressed in the following form

$$E = E^{\Theta} - \frac{59.1}{n} \log \frac{[TRX\text{-}(SH)^2]}{[TRX\text{-}SS]} \quad \text{mV} \qquad 25^{\circ}\,C, pH = 7 \tag{24}$$

Redox Environment of the Cell

Schafer and Buettner recently suggested that the redox state of a redox couple (for example α-T-OH/α-T-O$^{\bullet}$ or AscH^{-}/ Asc$^{\bullet-}$) is defined by the half-cell reduction potential and the reducing capacity of that couple (*1*). The redox state of all redox couples contributes to the redox environment of the cell.

The redox state of a biological system is given by the "total number of electrons" and is kept within a narrow range under normal conditions – in analogy according to which a biological system regulates its pH. Oxidative stress results in alterations of the redox state. A 30 mV change in the redox state means a 10-fold change in the ratio between reductant and oxidant (*1*).

Mitochondria use more than 90% of the cellular oxygen consumption. The mitochondrial electron transport chain is represented by a series of redox

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

reactions in which four electrons are transferred to oxygen which is subsequently reduced to water. However, in the course of the electron transport chain a small amount of dioxygen is reduced to superoxide by single electrons that leak (from 0.25 to 2%) from the electron transport chain (*22*). A small proportion of moving electrons that are not used for the energy production but are shunted to oxygen to form superoxide radical anions (and hydrogen peroxide) represents initiating species of redox cascades functioning in biological systems (*23*). Under physiological conditions, the steady state levels of superoxide and hydrogen peroxide molecules are very low. Hydrogen peroxide is an oxidant molecule participating in two-electron oxidation, typical for altering the redox equilibrium of thiol molecules activating thus the signalling pathways responsible for cell differentiation or alternatively at high flux cell apoptosis and necrosis (*24*).

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

Glutathione is the master antioxidant and smallest intracellular non-protein thiol molecule in the cells. Glutathione together with thioredoxin, which is represented by a group of small redox proteins are both responsible for the maintenance of the cellular redox state (*25*).

The thioredoxins are more specific than the glutathione system (*26*). Thioredoxins are known to react with approximately twenty biomolecules that are involved in various regulatory and catalytic processes. The thioredoxins are more specific than the glutathione system. Thioredoxin functioning interferes with the transcription factors such as NF-kappaB, AP-1 and the glucocorticoid receptor. Thioredoxins are also known to regulate stress signalling pathways, such as stress kinases ASK1 (*27*).

Glutathione is the major soluble antioxidant in these cell compartments and occurs in the cytosol (1–10 mM), nuclei (3–12 mM), and mitochondria (5–10 mM). Since GSH is synthesized in the cytosol, its mitochondrial functioning requires the mitochondrial electroneutral antiport carrier. The transport of GSH has been documented by experiments in which externally added GSH is readily taken up by mitochondria, despite the ~8mM GSH present in the mitochondrial matrix (*28*). A higher concentration of glutathione in the cell is more protective to the cell and requires a higher oxidation stimulus to change the redox cellular environment. Thus when examining the redox-buffer capacity of the cell one should consider the total concentration of GSH. Concentrations of GSH vary from cell to cell, for example liver cells contain approximately 5 mM of GSH.

Glutathione action in the nucleus is substantiated by the maintenance of the redox state of critical protein sulfhydryls that are necessary for the repair of DNA. An oxidative environment leads to oxidation of protein sulfhydryls (protein-SH): two electron oxidation yields sulfenic acids (protein-SOH) and one-electron oxidation yields thiyl radicals (protein-$S^{\bullet}$) (see Figure 2) (*29*).

Glutathione accomplishes its protective role against deleterious effect of reactive oxygen species (ROS) by the following mechanisms (*30*): (i) GSH is involved in amino acid transport through the plasma membrane, (ii) GSH is a direct scavenger of ROS including singlet oxygen, (iii) GSH regenerates various antioxidants, including ascorbic acid and alpha-tocopherol from their radical forms back to their active forms and (iv) GSH is a cofactor of various antioxidant enzymes.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

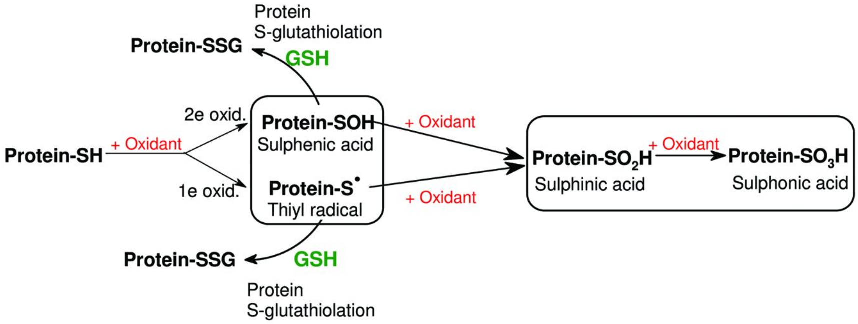


Figure 2. Protection of protein sulfhydryl groups by GHS.

The attack of proteins by ROS and reactive nitrogen species (RNS) affects practically all amino acid residues and polypeptide backbone (*2*). The attack of free radicals to proteins may lead to the oxidation of the amino acids. Cysteine and methionine residues of proteins are particularly susceptible to oxidation by ROS (*2*, *22*). The –SH (thiol or sulfhydryl) groups of cysteine aminoacids are the most vulnerable aminoacids to reversible and irreversible oxidation. The accumulation of oxidatively modified proteins plays an important role in the process of ageing.

A moderate oxidative environment confirmed by two-electron oxidation may lead to the formation of reversible sulfenic acid derivatives of cysteine (Protein-SOH, sulfenic acid) and via one electron oxidation to the thiyl radicals (protein-S•) (*31*). These partially oxidised products react with a tripeptide glutathione (present in the cytoplasm in millimolar concentration) and form S-glutathiolated protein (protein-SSG). If the process of oxidation of protein sulfhydryls is not interrupted by glutathione, further oxidation results in the formation of irreversibly oxidised products such as sulfinic (protein-SO_2H) and sulfonic (protein-SO_3H) acids (*22*, *31*). Thus when oxidative stress exceeds the potentially dangerous threshold, the process of S–glutathiolation triggers protective mechanisms against irreversible and deleterious oxidation of sensitive cysteine residues under conditions of oxidative stress.

The high abundance of reduced forms of both GSH and TRX is maintained by the activity of GSH-reductase and TRX-reductase, respectively (*32*). Both of these “redox buffering” thiol systems not only counteract intracellular oxidative stress and act as antioxidants in the cell, but they are also involved in cell signalling process (*3*, *33*). In addition to GSH and TRX, there are other both hydrophylic and lipophilic low molecular weight antioxidants, that when present at physiological concentration, can significantly contribute to overall ROS scavenging activity.

Triggering of the signalling processes in the cell require the action of the electrochemical potential gradients of the corresponding redox pairs. Experimental quantification of these gradients is now only in the early stage of development (*34*). The suitable systems in this respect are tumours characterized by the pronounced heterogeneity in their redox environment and partial pressure of oxygen, compared to healthy tissue.

Electron paramagnetic resonance (EPR) imaging is a suitable method for monitoring the redox status modulated by oxidative stress in vivo (*35*). EPR

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

spectroscopy, also referred to as Electron Spin Resonance (ESR) spectroscopy, is a nondestructive sensitive analytical technique which can be used for studies of species containing at least one unpaired electron (*36*). Such species are termed paramagnetic. In the EPR experiment, the sample, containing paramagnetic species with one or more unpaired electrons, is placed in a magnetic field, which removes the degeneracy of the various spin states of the paramagnetic particle. Transitions between the different spin states (alpha and beta) can then be induced by irradiation at the appropriate microwave frequency. The record of the absorption of the microwave radiation by the sample is the EPR spectrum. The EPR spectrum is sensitive to the nature and physical and chemical environment of the unpaired electrons and therefore it is very useful for the characterization of paramagnetic centers. The EPR spectroscopy has found numerous applications in chemistry, physics, biology and medicine. A very useful technique employed for the detection and characterization of short-lived radical species is EPR spin trapping (*37*). This technique is based on the application of a diamagnetic molecule (spin trap) which preferentially interacts with a reactive free radical to form a more stable spin adduct which can be detected by EPR technique. The most useful radical trap for the detection of oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline N-oxide (DMPO).

Low-frequency EPR and EPR-imaging techniques using nitroxide redox probes are in particular suitable for the monitoring of the cell redox environment status as well for the detection of the cellular hypoxic state that marks the onset of tumoural angiogenesis. In EPR experiments on tumour tissues of radiation-induced fibrosarcoma (RIF-1) tumour-bearing mice it has been confirmed that there is significant heterogeneity of redox status in the tumour tissue compared with normal tissue (*38*). EPR imaging has revealed that tumour tissues contain 4-fold higher concentrations of GSH levels compared with normal tissues. The significant heterogeneity of tumour cell redox status and the possibility of the fine tuning of the redox status of cancer cells may open new horizons in cancer therapy.

Acknowledgments

The authors appreciate funding by the Scientific Grant Agency of the Slovak Republic (Projects VEGA #1/0856/11) and by the Slovak Research and Development Agency of the Slovak Republic (Contract No.APVV-0202-10).

References

1. Schafer, F. Q.; Buettner, G. R. *Free Radical Biol. Med.* **2001**, *30*, 1191–1212.
2. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.
3. Dröge, W. *Physiol. Rev.* **2002**, *82*, 47–95.
4. Butler, J. In *Toxicology of the Human Environment*; Rhodes, C. J., Ed.; Taylor & Francis: London, 2000; pp 437–453.

5. Raff, L. M. *Principles of Physical chemistry*; Prentice Hall: New Jersey, 2001.
6. Schafer, F. Q.; Buettner, G. R. In *Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles*; Forman, H. J., Torres, M., Fukuto, J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2003; pp 1−14.
7. Koppenol, W. H.; Butler, J. *Adv. Free Radical Biol. Med.* **1985**, *1*, 91–131.
8. Pahari, A. K.; Chauhan, B. S. *Engineering Chemistry*; Firewall Media: New Delhi, India, 2007.
9. Daum, P.; Lenhard, J. R.; Rolison, D.; Murray, R. W. *J. Am. Chem. Soc.* **1980**, *102*, 4649–4653.
10. Wardman, P. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637–1755.
11. Rusling, J. F.; Miaw, C. L. *Environ. Sci. Technol.* **1989**, *23*, 476–479.
12. Alberty, R. A. *Thermodynamics of Biochemical Reactions*; John Wiley & Sons: New York, 2003.
13. van Boekel, M. J. A. S. *Kinetic Modeling of Reactions In Foods*; CRC Press: Boca Raton, FL, 2008
14. Buettner, G. R. *Arch. Biochem. Biophys.* **1993**, *300*, 535–543.
15. Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C. J.; Telser, J. *Mol. Cell. Biochem.* **2004**, *266*, 37–56.
16. Pilling, M. J. *Pure Appl. Chem.* **1992**, *64*, 1473–1480.
17. Forman, H. J.; Fukuto, J. M.; Torres, M. *Am. J. Physiol. Cell. Physiol.* **2004**, *287*, C246–C256.
18. Brumaghim, J. L.; Li, Y.; Henle, E.; Linn, S. *J. Biol. Chem.* **2003**, *278*, 42495–42504.
19. Veech, R. L.; Eggleston, L. V.; Krebs, HA. *Biochem. J.* **1969**, *155*, 609–619.
20. Kirlin, W. G.; Cai, J.; Thompson, S. A.; Diaz, D.; Kavanagh, T. J.; Jones, D. P. *Free Radical Biol. Med.* **1999**, *27*, 1208–1218.
21. Follmann, H.; Haeberlein, I. *BioFactors* **1995/1996**, *5*, 147–156.
22. Halliwell, B.; Gutteridge, J. M. C. In *Free Radicals in Biology and Medicine*; Oxford University Press: Cambridge, U.K., 2007.
23. Salvador, A.; Sousa, J.; Pinto, R. E. *Free Radical Biol. Med.* **2001**, *31*, 1208–1215.
24. Voehringer, D. W. *Free Radical Biol. Med.* **1999**, *27*, 945–950.
25. Forman, H. J.; Torres, M.; Fukuto, J. *Mol. Cell. Biochem.* **2002**, *234-235*, 49–62.
26. Matthews, J. R.; Wakasugi, N.; Virelizier, J. L.; Yodoi, J.; Hay, R. T. *Nucleic Acid Res.* **1992**, *20*, 821–3830.
27. Matsukawa, J.; Matsuzawa, A.; Takeda, K.; Ichijo, H. *J. Biochem.* **2004**, *136*, 261–265.
28. Shen, D.; Dalton, T. P.; Nebert, D. W.; Shertzer, H. G. *J. Biol. Chem.* **2005**, *280*, 25305–25312.
29. Ji, Y. B.; Akerboom, T. P. M.; Sies, H.; Thomas, J. A. *Arch. Biochem. Biophys.* **1999**, *362*, 67–78.
30. Masella, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; Giovannini, C. *J. Nutr. Biochem.* **2005**, *16*, 577–586.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

31. Dalle-Donne, I.; Rossi, R.; Colombo, G.; Giustarini, D.; Milzani, A. *Trends Biochem. Sci.* **2009**, *34*, 85–96.
32. Sun, Y.; Rigas, B. *Cancer Res.* **2008**, *68*, 8269–8277.
33. Thannickal, V. J.; Fanburg, B. L. *Am. J. Physiol.- Lung Cell. Mol. Physiol.* **2000**, *279*, L1005–L1028.
34. Ilangovan, G.; Li, H.; Zweier, J. L.; Kuppusamy, P. *Mol. Cell. Biochem.* **2002**, *234-235*, 393–398.
35. Kulkarni, A. C.; Bratasz, A.; Rivera, B.; Krishna, M. C.; Kuppusamy, P. *Isr. J. Chem.* **2008**, *48*, 27–31.
36. Stolc, S.; Valko, L.; Valko, M.; Lombardi, V. *Free Radical Biol. Med.* **1996**, *20*, 89–91.
37. Kasparova, S.; Brezova, V.; Valko, M.; Horecky, J.; Mlynarik, V.; Liptaj, T.; Vancova, O.; Ulicna, O.; Dobrota, D. *Neurochem. Int.* **2005**, *46*, 601–611.
38. Kuppusamy, P.; Li, H. Q.; Ilangovan, G.; Cardounel, A. J.; Zweier, J. L.; Yamada, K.; Krishna, M. C.; Mitchell, J. B. *Cancer Res.* **2002**, *62*, 307–312.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch003

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Chapter 4

Oxidative Stress in the Metabolism of Estrogens Leading to Cancer Initiation: Prevention by Specific Antioxidants

Eleanor G. Rogan*,[1,2] and Ercole L. Cavalieri[1,2]

[1]Department of Environmental, Agricultural and Occupational Health, College of Public Health, University of Nebraska Medical Center, Omaha, Nebraska 68198-4388
[2]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805
***E-mail:egrogan@unmc.edu**

Oxidative metabolism of the estrogens estrone (E_1) and estradiol (E_2) is the critical event in the initiation of cancer by estrogens. E_1 and E_2 are oxidized by cytochrome P450 (CYP) to the catechol estrogens 2-OHE$_1$(E$_2$) and 4-OHE$_1$(E$_2$) and then to the catechol estrogen quinones, which react with DNA to form estrogen-DNA adducts. The E_1(E_2)-3,4-quinones [E_1(E_2)-3,4-Q] react predominantly with DNA to form the depurinating adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua. The resulting apurinic sites in the DNA can generate mutations leading to the initiation of cancer. Estrogen metabolism becomes unbalanced when expression of the activating enzymes CYP19 (aromatase) and CYP1B1 is higher and expression of the protective enzymes catechol-*O*-methyltransferase and quinone reductase is lower. In this case, larger amounts of adducts are formed, and the risk of initiating cancer is greater. Women at high risk of developing breast cancer, or diagnosed with the disease, have higher levels of estrogen-DNA adducts than women at normal risk. These results and others in humans and cell culture indicate that formation of estrogen-DNA adducts is a critical event in the initiation of cancer. Two antioxidant compounds, *N*-acetylcysteine and resveratrol, efficiently block formation

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

of estrogen-DNA adducts and, thus, are promising agents to prevent cancer.

A weak mechanism of carcinogenesis for aromatic compounds, which includes the parent compound benzene, is the oxidative metabolism to phenols, followed by further hydroxylation to catechols and then oxidation to *ortho*-quinones (*1*). The electrophilic quinone compounds can react with DNA via 1,4-Michael addition, with predominant formation of depurinating adducts at the N-3 of adenine (Ade) and the N-7 of guanine (Gua) (Figure. 1). This mechanism of metabolic activation can occur with benzene (*2*, *3*), naphthalene (*4*, *5*), the natural estrogens estrone (E_1) and estradiol (E_2) (*6–9*) and the synthetic estrogens hexestrol (*10*, *11*) and diethylstilbestrol (DES) (Figure 2) (*12*). The apurinic sites formed by loss of these adducts from DNA can generate the critical mutations leading to the initiation of cancer (*13–15*).

For the estrogens, overwhelming evidence for this mechanism of carcinogenesis has been derived from experiments on estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity. In addition, unbalanced metabolism of estrogens has been shown to be the factor that renders the estrogens weak carcinogenic compounds. The predominant pathway that leads to the initiation of cancer is formation of $E_1(E_2)$-3,4-quinones [$E_1(E_2)$-3,4-Q] and reaction of this electrophilic compound with DNA to form the depurinating 4-OH$E_1(E_2)$-1-N3Ade and 4-OH$E_1(E_2)$-1-N7Gua adducts (Figure 1) (*9*, *16*). Error-prone repair of the resulting apurinic sites leads to mutations that can initiate cancer (*13–15*).

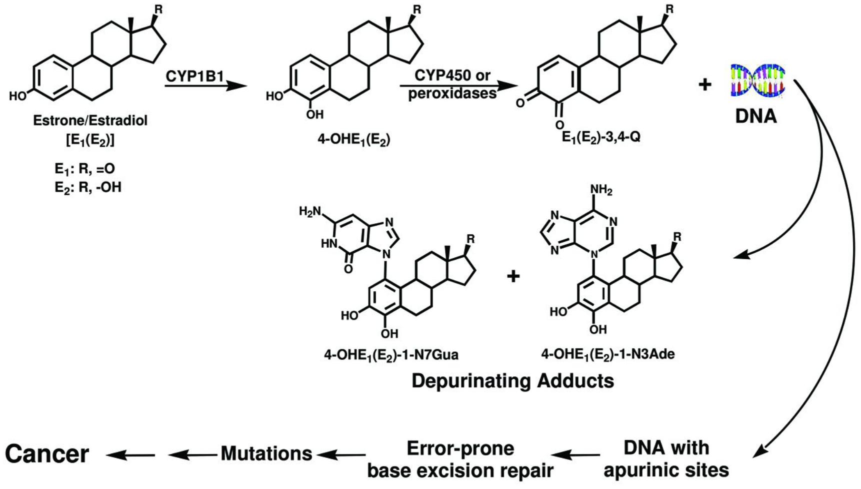

CYP1B1
CYP450 or peroxidases
Estrone/Estradiol
[E₁(E₂)]
E₁: R, =O
E₂: R, -OH
4-OHE₁(E₂)
E₁(E₂)-3,4-Q
DNA
4-OHE₁(E₂)-1-N7Gua
4-OHE₁(E₂)-1-N3Ade
Depurinating Adducts
Cancer
Mutations
Error-prone base excision repair
DNA with apurinic sites

Figure 1. Major metabolic pathway in cancer initiation by estrogens.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

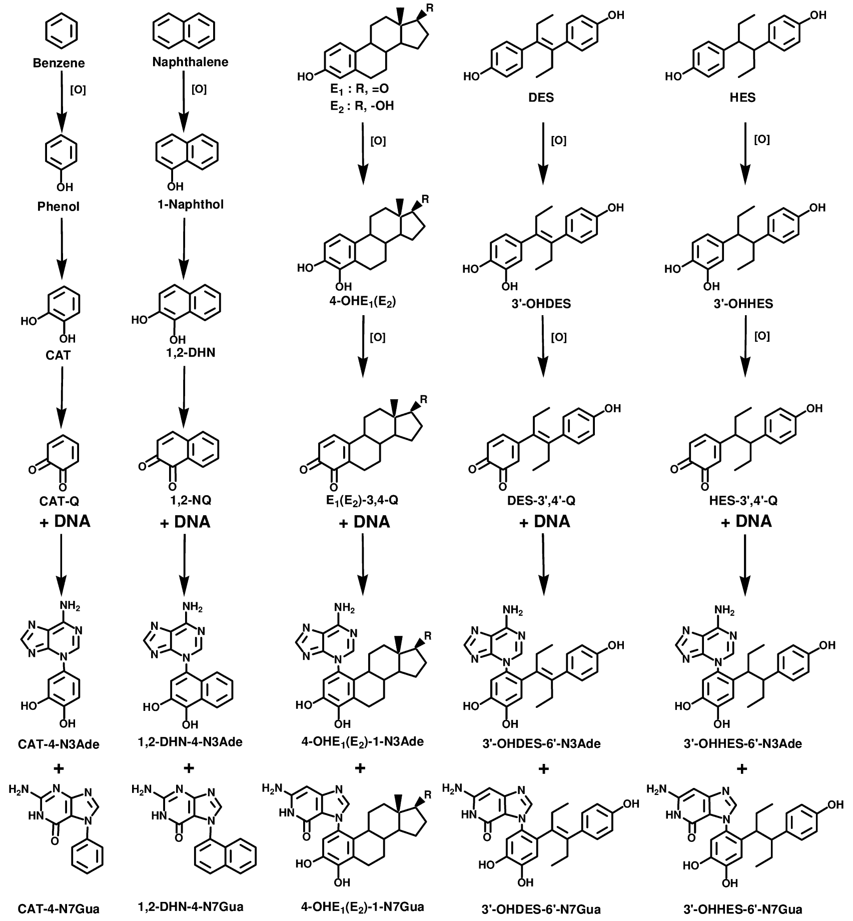

Benzene
[O]
Phenol
CAT
CAT-Q
+ DNA
CAT-4-N3Ade
+
CAT-4-N7Gua
Naphthalene
1-Naphthol
1,2-DHN
1,2-NQ
1,2-DHN-4-N3Ade
1,2-DHN-4-N7Gua
E1 : R, =O
E2 : R, -OH
4-OHE1(E2)
E1(E2)-3,4-Q
4-OHE1(E2)-1-N3Ade
4-OHE1(E2)-1-N7Gua
DES
3'-OHDES
DES-3',4'-Q
3'-OHDES-6'-N3Ade
3'-OHDES-6'-N7Gua
HES
3'-OHHES
HES-3',4'-Q
3'-OHHES-6'-N3Ade
3'-OHHES-6'-N7Gua

Figure 2. Common mechanism of metabolic activation to form depurinating DNA adducts for benzene, naphthalene, estrogens (E_1/E_2), DES and hexestrol. 1,2-DHN, 1,2-dihydroxynaphthalene; 1,2-NQ, 1,2-naphthoquinone; HES, hexestrol.

Estrogen Metabolism

Aromatization of 4-androstene-3,17-dione and testosterone is catalyzed by the enzyme cytochrome P450 (CYP)19 (aromatase) to produce E_1 and E_2, respectively (Figure 3). Excess estrogen is stored as E_1-sulfate. Estrogens are metabolized by two major pathways. One is 16α-hydroxylation (not shown in Figure 3), and the other is the catechol estrogen pathway in which E_1 and E_2 are hydroxylated at the 2- or 4- position. CYP1A isoforms preferentially hydroxylate the estrogens at the 2-position, whereas CYP1B1 catalyzes almost exclusively the formation of 4-OHE$_1$(E$_2$) (*17–19*). The catechol estrogens are inactivated, especially in the liver, by conjugating reactions such as glucuronidation and

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

sulfation (not shown in Figure 3). The most common conjugating pathway in extrahepatic tissues, however, is *O*-methylation of the hydroxyl group, catalyzed by catechol-*O*-methytransferase (COMT) (*20*). If the activity of COMT is low, methoxylation can be insufficient, and competitive oxidation of 2-OHE$_1$(E$_2$) and 4-OHE$_1$(E$_2$) can lead to formation of semiquinones and quinones by CYP or peroxidases (Figure 3).

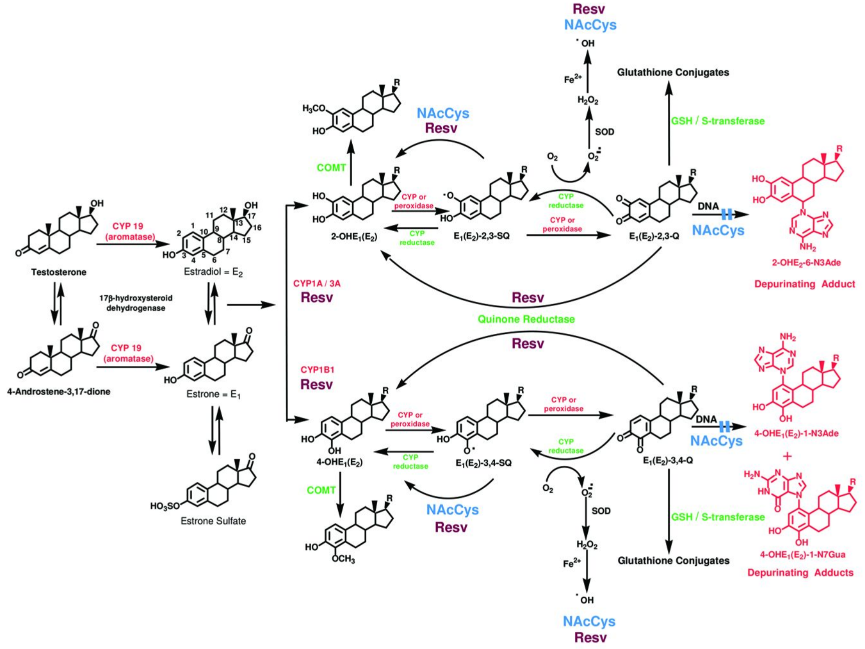

Resv
NAcCys
•OH
Fe2+
H2O2
SOD
O2
O2•
Glutathione Conjugates
GSH / S-transferase
H3CO
HO
NAcCys
Resv
COMT
CYP or peroxidase
CYP reductase
CYP or peroxidase
2-OHE1(E2)
E1(E2)-2,3-SQ
E1(E2)-2,3-Q
DNA
NAcCys
2-OHE2-6-N3Ade
Depurinating Adduct
Testosterone
CYP 19 (aromatase)
Estradiol = E2
17β-hydroxysteroid dehydrogenase
4-Androstene-3,17-dione
CYP 19 (aromatase)
Estrone = E1
Estrone Sulfate
HO3SO
CYP1A / 3A
Resv
Resv
Quinone Reductase
Resv
CYP1B1
Resv
CYP or peroxidase
CYP or peroxidase
CYP reductase
CYP reductase
4-OHE1(E2)
E1(E2)-3,4-SQ
E1(E2)-3,4-Q
COMT
OCH3
NAcCys
Resv
DNA
NAcCys
4-OHE1(E2)-1-N3Ade
+
4-OHE1(E2)-1-N7Gua
Depurinating Adducts
GSH / S-transferase
Glutathione Conjugates
NAcCys
Resv

Figure 3. Formation, metabolism and DNA adducts of estrogens. Activating enzymes and depurinating DNA adducts are in red and protective enzymes are in green. N-Acetylcysteine (NAcCys, shown in blue) and resveratrol (Resv, burgundy) indicate the various points where NAcCys and Resv could improve the balance of estrogen metabolism and minimize formation of depurinating estrogen-DNA adducts.

Estrogen semiquinones can be oxidized to quinones by molecular oxygen (Figure 3). In turn, the estrogen quinones can be reduced to semiquinones by CYP reductase. This reaction completes redox cycling. In this process, the molecular oxygen is reduced to superoxide anion radical, which is converted to H_2O_2. In the presence of Fe^{2+}, H_2O_2 yields reactive hydroxyl radicals. Formation of lipid hydroperoxides can occur as the first damage by hydroxyl radicals. The lipid hydroperoxides can then act as unregulated cofactors of CYP; this lack of regulation can generate an abnormal increase in the oxidation of catechol estrogens to quinones. Thus, efficient redox cycling can generate abundant catechol estrogen quinones, which are the ultimate carcinogenic metabolites of estrogens. The catechol estrogen quinones can be inactivated by conjugation with glutathione (GSH, Figure 3). A second inactivating pathway for the quinones is their reduction to catechol by quinone reductase (NQO1 and NQO2) (*21*, *22*). This protective enzyme can be induced by a variety of compounds (*23*). If all of the protective processes become insufficient, the catechol estrogen quinones can react with DNA to yield predominantly depurinating DNA adducts.

Depurinating Estrogen-DNA Adducts

There are two types of carcinogen-DNA adducts: stable adducts that remain in DNA unless removed by repair and depurinating adducts that separate from DNA by destabilization of the glycosyl bond. Stable adducts are formed when a carcinogenic compound reacts with the exocyclic amino group of Ade or Gua, whereas formation of adducts at the N-3 and N-7 of Ade or the N-7 of Gua destabilizes the glycosyl bond, with subsequent depurination of the adduct from DNA (*6–9*).

Evidence that depurinating DNA adducts play a major role in cancer initiation derives from the correlation between the levels of depurinating aromatic hydrocarbon-DNA adducts and oncogenic Harvey (H)-*ras* mutations in mouse skin papillomas (*24*). A similar correlation between the sites of formation of depurinating DNA adducts and H-*ras* mutations was observed in mouse skin and rat mammary gland treated with E_2-3,4-Q (*13*, *14*).

Reaction of $E_1(E_2)$-3,4-Q with DNA by 1,4-Michael addition forms the depurinating adducts 4-OH$E_1(E_2)$-1-N3Ade and 4-OH$E_1(E_2)$-1-N7Gua in 99% yield, with stable adducts comprising the remaining 1% (*6–9*). Instead, reaction of $E_1(E_2)$-2,3-Q forms much lower levels of 2-OH$E_1(E_2)$-6-N3Ade adducts (*9*) by 1,6-Michael addition after tautomerization of the quinone to the $E_1(E_2)$-2,3-Q methide (*25*). The levels of the depurinating DNA adducts formed by the catechol estrogen quinones agree with the greater carcinogenic activity of 4-OH$E_1(E_2)$ compared to the lack of carcinogenic activity of 2-OH$E_1(E_2)$ in several animal models (*26–29*). The critical role of depurinating DNA adducts and the apurinic sites they generate has also been observed in the mutagenicity of E_2-3,4-Q *in vivo* (*13*, *14*).

Mutagenicity of Estrogens

Until ten years ago, estrogens were thought to be non-mutagenic because mutations were not observed in the assays employed. This has been the cornerstone of denying the genotoxicity of estrogens (*30*). More recently, the major pathways of metabolic activation of estrogens have been elucidated, and more appropriate and sensitive assay conditions have been used. Thus, both 4-OHE_2 and E_2-3,4-Q have been shown to be mutagenic (*13*, *14*, *31*). The mutagenicity of 4-OHE_2 when it can be metabolized to E_2-3,4-Q and the mutagenicity of the quinone itself provide the necessary evidence to demonstrate that estrogens are, indeed, genotoxic compounds.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

The first demonstration of the mutagenicity of E_2-3,4-Q was obtained by treating the dorsal skin of female SENCAR mice and determining both the estrogen-DNA adducts formed and the H-*ras* mutations induced in the skin (*13*). Similarly, treatment of the mammary glands of female ACI rats by intramammillary injection of E_2-3,4-Q generated both estrogen-DNA adducts and H-*ras* mutations (*14*). The mutagenic activity of 4-OHE_2 was demonstrated in Big Blue rats, which are Fischer 344 rats with about 80 copies of the λ-LIZ vector in each cell. This transgene is not expressed and has no effect on the biochemistry or physiology of the cells. After 20 weeks of treatment with 4-OHE_2, a statistically significant number of mutations was detected in the *cII* gene (*15*). Finally, treatment of cultured Big Blue rat2 embryonic cells, which contain approximately 60 copies of the λ-LIZ vector, with 4-OHE_2 or E_2-3,4-Q generated mutations (*31*).

In all of these models, approximately equal amounts of the 4-OHE_1(E_2)-1-N3Ade and 4-OHE_1(E_2)-1-N7Gua adducts were detected, but mutations were predominantly observed at A.T base pairs. This finding may result from the instantaneous depurination of the 4-OHE_1(E_2)-1-N3Ade adducts and much slower depurination of the 4-OHE_1(E_2)-1-N7Gua adducts (*9*, *10*). The rapid depurination of the N3Ade adducts may favor error-prone repair of the apurinic sites and generation of mutations, while the slower depurination of N7Gua adducts may allow error-free repair of the DNA.

Transformation of Cells by Estrogens

Studies with cultured breast epithelial cells from women or mice have provided evidence that initiation of cancer occurs by formation of estrogen-DNA adducts. The MCF-10F cell line is an immortalized non-transformed estrogen receptor-α-negative human cell line. When these cells are treated with E_2 or 4-OHE_2, the depurinating estrogen-DNA adducts are formed (*32–34*). Treatment with E_2 or 4-OHE_2 at doses of 0.007-3.5 nM produces transformation of these cells, as detected by their ability to form colonies in soft agar (*35*, *36*). The presence of the antiestrogen tamoxifen or ICI-182,780 does not prevent this transformation (*37*). These changes are induced to a much smaller extent by 2-OHE_2. These results indicate that transformation is determined by genotoxic effects of the estrogen metabolite. When estrogen-transformed MCF-10F cells, which were selected by their invasiveness, were implanted into severely compromised immune-deficient mice, the cells induced tumors (*38*). These results demonstrate that human breast epithelial cells lacking estrogen receptor-α are transformed by the genotoxic effects of estrogen metabolites. Thus, these results support the hypothesis that formation of depurinating estrogen-DNA adducts is the critical event in the initiation of cancer by estrogens.

Similarly, the immortalized, normal mouse mammary cell line E6 also forms depurinating estrogen-DNA adducts and is transformed to grow in soft agar by a single treatment with 4-OHE_2 or E_2-3,4-Q (*39*). These results demonstrate that transformation of breast cells by estrogen genotoxicity occurs in both humans and animals.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Carcinogenicity of Estrogens

The two catechol estrogens 4-OHE$_1$(E$_2$) and 2-OHE$_1$(E$_2$) were first tested for carcinogenic activity by subcutaneous implantation into male Syrian golden hamsters. The 4-OHE$_1$(E$_2$) were carcinogenic, while the 2-OHE$_1$(E$_2$) were not (*26*, *27*). The two catechol estrogens were also tested in CD-1 mice by injection of the compound into newborns. Once again, the 4-catechol estrogen induced uterine adenocarcinomas, whereas the 2-catechol estrogen was borderline active (*28*). These results are consistent with the structure of the two catechols. The 4-catechol estrogens, when oxidized to their quinones, produce an electrophilic species that reacts very strongly with DNA by protonated 1,4-Michael addition (*40*), whereas the catechol estrogen-2,3-quinones react with DNA by 1,6-Michael addition via an intermediate quinone methide (*25*). For this reason, E$_1$(E$_2$)-3,4-Q react with DNA to a much greater extent than the E$_1$(E$_2$)-2,3-Q (*9*), forming 97% of the depurinating adducts found in humans (*41*).

Studies of transgenic mice with estrogen receptor-α knocked out, ERKO/*wnt-1* mice, provide further important evidence demonstrating the role of estrogen genotoxicity in the initiation of cancer. Despite the absence of estrogen receptor-α, mammary tumors develop in 100% of female ERKO/*wnt-1* mice, driven by the *wnt-1* transgene (*42*, *43*). The protective methoxy estrogen conjugates were not found in the mammary tissue of female ERKO/*wnt-1* mice, but 4-OHE$_1$(E$_2$) and estrogen-GSH conjugates were detected (*44*). These results indicate that estrogen metabolism in these mice is unbalanced toward excess activating pathways and limited protective pathways. When the mice were implanted with E$_2$ following ovariectomy at 15 days of age to remove their major source of estrogens, the E$_2$-treated mice developed mammary tumors in a dose-dependent manner (*45*, *46*). The mammary tumors developed even in the presence of the implanted anti-estrogen ICI-182,780 (*47*). These results provide strong evidence for the critical role of estrogen genotoxicity in tumor initiation.

Imbalances in Estrogen Metabolism

Initiation of cancer by estrogens occurs when a relatively large amount of E$_1$(E$_2$)-3,4-Q reacts with DNA, contributing about 97% of the adducts. The E$_1$(E$_2$)-2,3-Q contributes minimally to the formation of estrogen-DNA adducts because this electrophile reacts weakly with the N-3 of Ade to form an adduct (*9*). A large amount of the reaction of the quinones with DNA is due to oxidative stress, namely, the oxidative events that give rise to the catechol quinones in great abundance. These oxidative events start with the formation of estrogens from androgens, catalyzed by CYP19 (aromatase) (Figure 3). When this enzyme is over-expressed, a large amount of estrogen is produced and stored as E$_1$-sulfate (Figure 3). This E$_1$-sulfate is transformed to E$_1$ and E$_2$ by sulfatase when needed; however, if the sulfatase is not regulated it can produce excess estrogens.

In general, formation of 4-OHE$_1$(E$_2$) is specifically catalyzed by CYP1B1 (*17–19*). Overexpression of this enzyme can generate a large amount of these catechol estrogens. If the formation of catechols is very abundant and the activity of COMT is insufficient, the oxidation of catechols to semiquinones and quinones becomes competitive (Figure 3). The quinones, $E_1(E_2)$-2,3-Q and $E_1(E_2)$-3,4-Q, can be conjugated with GSH or reduced back to catechols by the enzyme quinone reductase (*21*, *22*). Once again, if these two protective events are insufficient, the quinones can react with DNA (Figure 3). In fact, breast tissue from women with breast cancer has high levels of expression of the activating enzymes CYP19 (aromatase) and CYP1B1 and low expression of the protective enzymes COMT and NQO1, whereas women who do not have breast cancer have the opposite pattern of enzyme expression (*48*).

A relatively high amount of the depurinating 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1 N7Gua adducts represents an index of unbalanced estrogen metabolism. This occurs only when the oxidative events overcome the protective events.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

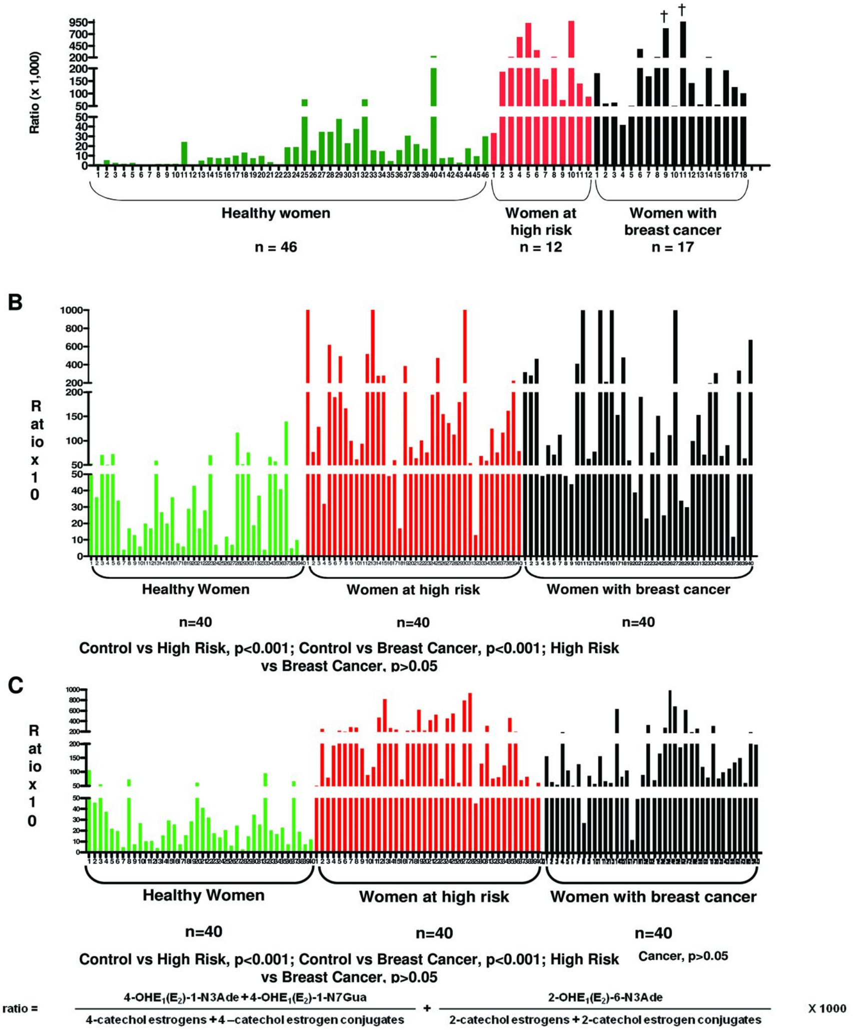
Levels of Estrogen-DNA Adducts in People with and without Cancer

While most of us metabolize estrogens to products that are easily excreted from the body, people at risk for cancer metabolize estrogens to increased levels of $E_1(E_2)$-3,4-Q, which can react with DNA to form the depurinating adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1 N7Gua. These adducts are shed from DNA, and the resulting apurinic sites can be unfaithfully repaired to generate mutations leading to cancer (*13–15*, *49*). After the depurinating adducts are released from DNA, they travel out of cells and tissues into the bloodstream and are excreted in urine. Thus, they can be identified and quantified as biomarkers of risk of developing cancer (*41*, *50–53*).

High levels of depurinating estrogen-DNA adducts have been detected in analyses of urine and serum from women that are at high risk for breast cancer or have been diagnosed with the disease (Figure 4) (*41*, *50*, *54*). A spot urine sample and/or a serum sample was obtained from women at normal risk for breast cancer, high risk for breast cancer [Gail Model score >1.66% (*55*)] and women diagnosed with breast cancer. After partial purification of an aliquot by solid phase extraction, each sample was analyzed for 40 estrogen metabolites, conjugates and depurinating DNA adducts by using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS).

The risk of developing breast cancer was measured as the ratio of depurinating estrogen-DNA adducts to their respective estrogen metabolites and conjugates (Figure 4) because this ratio indicates the degree of imbalance in a person's estrogen metabolism. Typically, the high ratio for women at high risk or diagnosed with breast cancer results from a high level of adducts and low levels of metabolites and conjugates. In some of these women, however, the adduct level is not high, but the levels of metabolites and conjugates are very low, presumably because a high proportion of the metabolites was converted to adducts. The DNA adducts formed by $E_1(E_2)$-3,4-Q are predominant (97%) in this ratio, whereas the adducts formed by $E_1(E_2)$-2,3-Q are minimal (3%) (*41*, *50*, *54*). The typically low ratio in women at normal risk for breast cancer indicates that their estrogen metabolism is balanced and they form relatively few estrogen-DNA adducts.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004



$$\text{ratio} = \frac{\text{4-OHE}_1(\text{E}_2)\text{-1-N3Ade} + \text{4-OHE}_1(\text{E}_2)\text{-1-N7Gua}}{\text{4-catechol estrogens} + \text{4-catechol estrogen conjugates}} + \frac{\text{2-OHE}_1(\text{E}_2)\text{-6-N3Ade}}{\text{2-catechol estrogens} + \text{2-catechol estrogen conjugates}} \quad \text{X 1000}$$

Figure 4. Depurinating estrogen-DNA adducts in (A) urine of healthy women, high-risk women and women with breast cancer – first study (41); (B) urine of healthy women, high-risk women and women with breast cancer – second study (50); and (C) serum of healthy women, high-risk women and women with breast cancer (54).

In two studies of women at normal risk, high risk or diagnosed with breast cancer, highly significant differences were observed in the relative levels of estrogen-DNA adducts when urine samples from normal-risk women were compared to women at high risk or with the disease (Figure 4) (*41*, *50*). Similar differences were observed when serum samples from the same women were analyzed (*54*). Subject characteristics did not affect the highly significant differences. Thus, these studies demonstrate that unbalanced estrogen metabolism leading to increased levels of estrogen-DNA adducts is associated with high risk of developing breast cancer.

Similarly, analysis of urine samples from men with and without prostate cancer showed that men with the disease have high levels of estrogen-DNA adducts compared to healthy control men (Figure 5) (*51*, *52*). In addition, urine from men diagnosed with non-Hodgkin lymphoma has high levels of estrogen-DNA adducts compared to healthy controls (*53*). These results indicate that formation of estrogen-DNA adducts is associated with several types of cancer in both men and women, and the adducts could play a critical role in the etiology of these types of cancer.

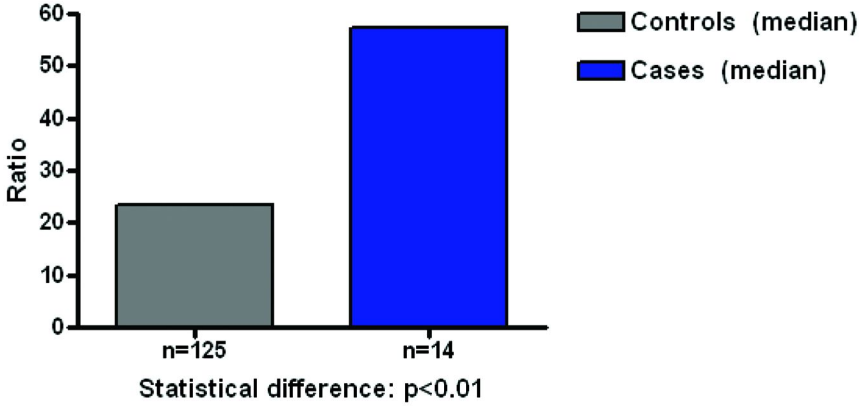


Figure 5. Levels of estrogen-DNA adducts in urine samples from men with and without prostate cancer (52).

Cancer Prevention by Antioxidant Compounds

The estrogens E_1 and E_2 are obtained biosynthetically from 4-androstene-3,17-dione and testosterone in a reaction catalyzed by CYP19 (aromatase) (Figure 3). In the catechol estrogen pathway of metabolism, E_1 and E_2 are oxidized to 2-$OHE_1(E_2)$ and 4-$OHE_1(E_2)$. CYP1A and CYP3A yield preferentially the 2-$OHE_1(E_2)$, whereas CYP1B1 forms almost exclusively 4-$OHE_1(E_2)$. The antioxidants that we have selected exert their influence at various levels of oxidation of the catechol estrogens, namely, formation of semiquinones and then quinones, followed by reaction of the quinones with DNA to form depurinating adducts. The reaction of $E_1(E_2)$-2,3-Q is rather ineffective and contributes minimally to the reaction with DNA to form 2-$OHE_1(E_2)$-6-N3Ade, which are only 2-3% of the adducts (*9*). Ninety-seven percent of the adducts arise from the reaction of $E_1(E_2)$-3,4-Q with DNA.

We have selected antioxidants for keeping the metabolism of estrogens in balance, resulting in minimal amounts of formation of catechol estrogen quinones and their reaction with DNA. Reaction of E_2-3,4-Q with DNA in the presence of one of five antioxidants, namely, GSH, *N*-acetylcysteine (NAcCys), dihydrolipoic acid, melatonin or resveratrol, showed that only GSH, NAcCys and dihydrolipoic

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

acid react with the quinone to inhibit its reaction with DNA (*56*). NAcCys has slightly better antioxidant properties than GSH and dihydrolipoic acid, which are very similar (Figure 6A). Resveratrol and melatonin, which do not have in their structure a functional group that reacts with quinones, exhibit no antioxidant properties in the reaction of the quinone with DNA.

When the same reaction is carried out using 4-OHE$_2$ activated by lactoperoxidase, the best antioxidant is resveratrol, followed by NAcCys, dihydrolipoic acid, GSH and melatonin (Figure 6B) (*56*). The inhibition by resveratrol and melatonin presumably arises from their ability to reduce the E$_2$-3,4-semiquinone back to 4-OHE$_2$ (*56*). GSH, NAcCys and dihydrolipoic acid inhibit the formation of adducts from activated 4-OHE$_2$ about 5 to 15% more than the formation of adducts from E$_2$-3,4-Q (Figure 6). In addition to reacting with E$_2$-3,4-Q, NAcCys is known to reduce the E$_2$-3,4-semiquinone back to 4-OHE$_2$ (*57*), and we speculate that dihydrolipoic acid and GSH can also carry out this reduction.

Ample evidence indicates that the initiation of breast, prostate and other human cancers occurs when the metabolism in the catechol estrogen pathway is unbalanced (*1*, *15*). In this case, an excessive amount of catechol estrogen quinones, in particular E$_1$(E$_2$)-3,4-Q, are formed (Figure 3). Under these circumstances, the quinones react with DNA to form more depurinating adducts, 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua. This event represents the initiation of cancer by estrogens.

The unbalance in estrogen metabolism is mainly created by the two activating enzymes, CYP19 (aromatase) and CYP1B1. This unbalance also can occur when the protective enzymes COMT and/or quinone reductase are weakly expressed or deficient in catalytic activity (*48*). COMT converts catechol estrogens to their methoxy derivatives, thereby impeding further oxidation of catechol estrogens to their semiquinones and quinones (Figure 3). Quinone reductases, NQO1 and NQO2, manifest their protective action by reducing the electrophilic, reactive quinones to catechols (*21*, *22*). Reaction of catechol estrogen quinones with GSH, with or without catalytic input by glutathione-*S*-transferase, represents another factor in balanced estrogen metabolism.

Two antioxidants have demonstrated great efficiency in keeping the catechol estrogen oxidative pathway balanced in the MCF-10F human breast epithelial cell line and in the E6 mouse mammary cell line (*39*, *58–61*). The antioxidant effect of NAcCys in reducing formation of estrogen-DNA adducts in MCF-10F cells treated with 4-OHE$_2$ (*60*) is due to the reaction of NAcCys with E$_2$-3,4-Q and the reduction of E$_2$-3,4-semiquinone to 4-OHE$_2$ (*57*). Resveratrol also reduces E$_2$-3,4-semiquinone to 4-OHE$_2$ (*58*, *59*). In addition, resveratrol induces NQO1, which catalyes the reduction of E$_2$-3,4-Q to 4-OHE$_2$ (*21*), thereby limiting reaction of the quinone with DNA (Figure 3). Resveratrol displays another important effect by modulating the action of CYP1B1 if it is overexpressed (*58*). When MCF-10F cells are treated with 4-OHE$_2$ plus NAcCys and resveratrol mixed together, the two antioxidants display an additive effect in reducing the formation of estrogen-DNA adducts by the cells (Figure 7) (*61*). At low concentrations, the two antioxidants inhibit formation of estrogen-DNA adducts similarly, but at higher concentrations, the effect of resveratrol is 50% greater than that of NAcCys (*61*).

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

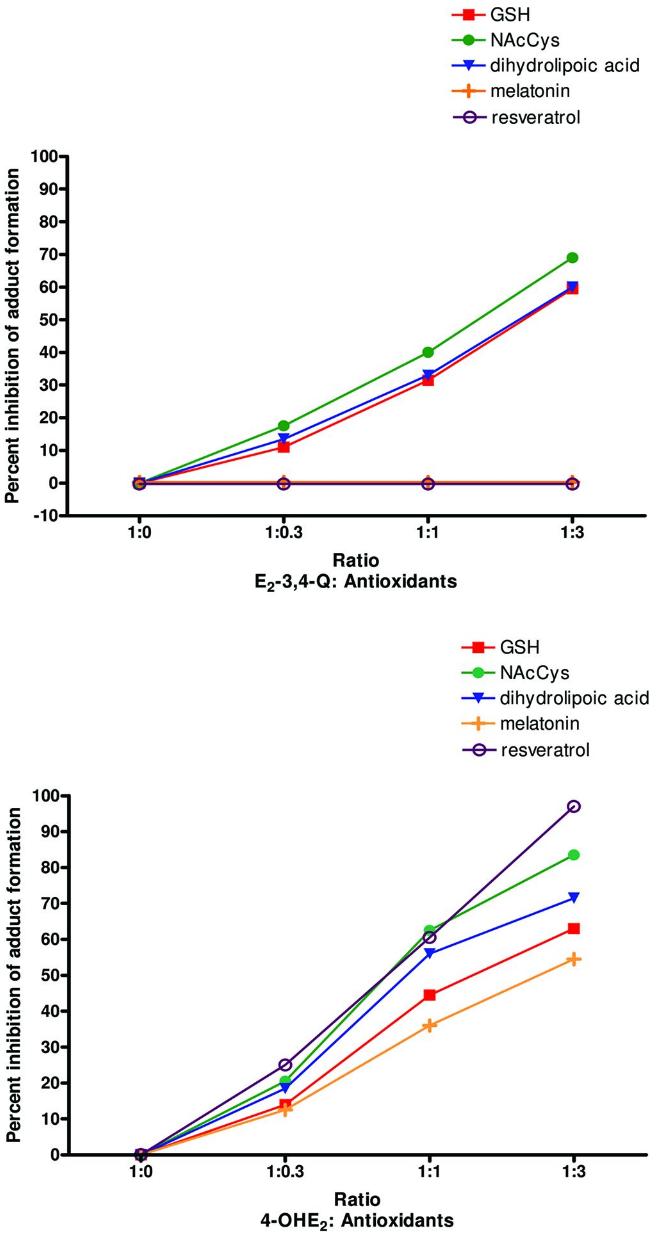
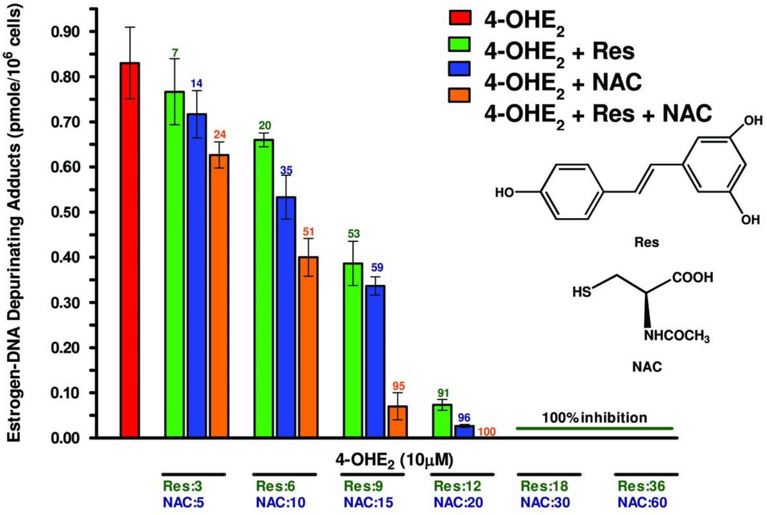


Figure 6. Effect of the antioxidants GSH, NAcCys, dihydrolipoic acid, melatonin and resveratrol on the reaction of (A) E_2-3,4-Q and (B) enzyme-activated 4-OHE_2 with DNA in vitro (56).

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004



The numbers on bars are % inhibition of depurinating adducts, compared to 4-OHE$_2$ alone treatment value.

Figure 7. Effects of NAcCys, resveratrol, or NAcCys plus resveratrol on the formation of depurinating estrogen–DNA adducts in MCF-10F cells treated with 4-OHE$_2$. NAC, NAcCys; Res, resveratrol (61).

Resveratrol was also found to inhibit the malignant transformation of MCF-10F cells treated with E_2 (*58*). NAcCys was found to inhibit not only formation of estrogen-DNA adducts, but also transformation of E6 mouse mammary cells treated with 4-OHE$_2$ or E_2-3,4-Q (*39*).

Thus, the use of these two antioxidants serves to re-establish and/or maintain balanced estrogen metabolism. This effect reduces DNA damage and the resulting mutations that can lead to the initiation of cancer. Therefore, NAcCys and resveratrol are promising candidates for the prevention of estrogen-initiated cancers in human beings.

Acknowledgments

Our progress in research on the etiology and prevention of cancer is due to the efforts, dedication, accomplishments and creativity of the fine scientists who have worked with us over the years. We express our gratitude to Drs. D. Chakravarti, N. Gaikwad, K.-M. Li, M. Saeed, S. Singh, D. Venugopal and M. Zahid, our graduate students F. Lu and L. Yang, and our technical support P. Mailander and S. Higginbotham. Preparation of this article was supported by Prevention LLC. Core support at the Eppley Institute was supported by grant P30 36727 from the National Cancer Institute.

References

1. Cavalieri, E.; Rogan, E. *J. Steroid Biochem. Mol. Biol.* **2011**, DOU: 10.1016/j.jsbmb.2011.03.008.
2. Cavalieri, E. L.; Li, K.-M.; Balu, N.; Saeed, M.; Devanesan, P.; Higginbotham, S.; Zhao, J.; Gross, M. L.; Rogan, E. *Carcinogenesis* **2002**, *23*, 1071–1077.
3. Zahid, M.; Saeed, M.; Rogan, E. G.; Cavalieri, E. L. *Free Radical Biol. Med.* **2010**, *48*, 318–324.
4. Saeed, M.; Higginbotham, S.; Rogan, E.; Cavalieri, E. *Chem-Biol. Interact.* **2007**, *165*, 175–188.
5. Saeed, M.; Higginbotham, S.; Gaikwad, N.; Chakravarti, D.; Rogan, E.; Cavalieri, E. *Free Radical Biol. Med.* **2009**, *47*, 1075–1081.
6. Stack, D.; Byun, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. *Chem. Res. Toxicol.* **1996**, *9*, 851–859.
7. Cavalieri, E. L.; Stack, D. E.; Devanesan, P. D.; Todorovic, R.; Dwivedy, I.; Higginbotham, S.; Johansson, S. L.; Patil, K. D.; Gross, M. L.; Gooden, J. K.; Ramanathan, R.; Cerny, R. L.; Rogan, E. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10937–10942.
8. Li, K.-M.; Todorovic, R.; Devanesan, P.; Higginbotham, S.; Köfeler, H.; Ramanathan, R.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. *Carcinogenesis* **2004**, *25*, 289–297.
9. Zahid, M.; Kohli, E.; Saeed, M.; Rogan, E.; Cavalieri, E. *Chem. Res. Toxicol.* **2006**, *19*, 164–172.
10. Saeed, M.; Zahid, M.; Gunselman, S. J.; Rogan, E.; Cavalieri, E. *Steroids* **2005**, *70*, 29–35.
11. Saeed, M.; Gunselman, S. J.; Higginbotham, S.; Rogan, E.; Cavalieri, E. *Steroids* **2005**, *70*, 37–45.
12. Saeed, M.; Rogan, E.; Cavalieri, E. *Int. J. Cancer* **2009**, *124*, 1276–1284.
13. Chakravarti, D.; Mailander, P.; Li, K.-M.; Higginbotham, S.; Zhang, H.; Gross, M. L.; Cavalieri, E.; Rogan, E. *Oncogene* **2001**, *20*, 7945–7953.
14. Mailander, P. C.; Meza, J. L.; Higginbotham, S.; Chakravarti, D. *J. Steroid Biochem.* **2006**, *101*, 204–215.
15. Cavalieri, E.; Chakravarti, D.; Guttenplan, J.; Hart, E.; Ingle, J.; Jankowiak, R.; Muti, P.; Rogan, E.; Russo, J.; Santen, R.; Sutter, T. *Biochem. Biophys. Acta, Rev. Cancer* **2006**, *1766*, 63–78.
16. Cavalieri, E.; Frenkel, K.; Liehr, J. G.; Rogan, E; Roy, D. In *JNCI Monograph: Estrogens as Endogenous Carcinogens in the Breast and Prostate*; Cavalieri, E, Rogan, E., Eds.; Estrogens as Endogenous Genotoxic Agents: DNA Adducts and Mutations; Oxford University Press: Oxford, U.K., 2000; pp 75−93.
17. Spink, D. C.; Hayes, C. L.; Young, N. R.; Christou, M.; Sutter, T. R.; Jefcoate, C. R.; Gierthy, J. F. *J. Steroid Biochem. Mol. Biol.* **1994**, *51*, 251–258.
18. Hayes, C. L.; Spink, D. C.; Spink, B. C.; Cao, J. Q; Walker, N. L.; Sutter, T. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9776–9781.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

19. Spink, D. C.; Spink, B. C.; Cao, J. Q.; DePasquale, J. A.; Pentecost, B. T.; Fasco, M. J.; Li, Y.; Sutter, T. R. *Carcinogenesis* **1998**, *19*, 291–298.
20. Männistö, P. T.; Kaakkola, S. *Pharmacol. Rev.* **1999**, *51*, 593–628.
21. Gaikwad, N. W.; Rogan, E. G.; Cavalieri, E. L. *Free Radical Biol. Med.* **2007**, *43*, 1289–1298.
22. Gaikwad, N. W.; Yang, L.; Rogan, E. G.; Cavalieri, E. L. *Free Radical Biol. Med.* **2009**, *46*, 253–262.
23. Talalay, P.; Dinkova-Kostova, A. T.; Holtzclaw, W. D. *Adv. Enzyme Regul.* **2003**, *43*, 121–134.
24. Chakravarti, D.; Pelling, J. C.; Cavalieri, E. L.; Rogan, E. G. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10422–10426.
25. Bolton, J. L.; Shen, L. *Carcinogenesis* **1998**, *17*, 925–929.
26. Liehr, J. G.; Fang, W. F.; Sirbasku, D. A.; Ari-Ulubelen, A. *J. Steroid Biochem.* **1986**, *24*, 353–356.
27. Li, J. J.; Li, S. A. *Fed. Proc.* **1987**, *46*, 1858–1863.
28. Newbold, R. R.; Liehr, J. G. *Cancer Res.* **2000**, *60*, 235–237.
29. Snten, R. J.; Yue, W.; Bocchinfuso, W.; Korach, K.; Wang, J.-P.; Rogan, E. G.; Li, Y.; Cavalieri, E.; Russo, J.; Devanesan, P.; Verderame, M. In *Advances in Endocrine Therapy of Breast Cancer*; Ingle, J. N., Dowsett, M., Eds.; Estradiol-Induced Carcinogenesis via Formation of Genotoxic Metabolites; Marcel Dekker: New York, 2004; pp 163−177.
30. Liehr, J. G. *Endocr. Rev.* **2000**, *21*, 40–54.
31. Zhao, Z.; Kosinska, W.; Khmelnitsky, M.; Cavalieri, E. L.; Rogan, E. G.; Chakravarti, D.; Sacks, P.; Guttenplan, J. B. *Chem. Res. Toxicol.* **2006**, *19*, 475–479.
32. Lu, F.; Zahid, M.; Saeed, M.; Cavalieri, E. L.; Rogan, E. G. *J. Steroid Biochem. Mol. Biol.* **2007**, *105*, 150–158.
33. Saeed, M.; Rogan, E.; Fernandez, S. V.; Sheriff, F.; Russo, J.; Cavalieri, E. *Int. J. Cancer* **2007**, *120*, 1821–1824.
34. Zahid, M.; Saeed, M.; Lu, F.; Gaikwad, N.; Cavalieri, E. L.; Rogan, E. G. *Free Radical Biol. Med.* **2007**, *43*, 1534–1540.
35. Russo, J.; Hasan Lareef, M.; Balogh, G.; Guo, S.; Russo, I. H. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 1–25.
36. Russo, J.; Russo, I. H. *Trends Endocrinol. Metab.* **2004**, *15*, 211–214.
37. Lareef, M. H.; Garber, J.; Russo, P. A.; Russo, I. H.; Heulings, R.; Russo, J. *Int. J. Oncol.* **2005**, *26*, 423–429.
38. Russo, J.; Fernandez, S. V.; Russo, P. A.; Fernbaugh, R.; Sheriff, F. S.; Lareef, H. M.; Garber, J.; Russo, I. H. *FASEB J.* **2006**, *20*, 1622–1634.
39. Venugopal, D.; Zahid, M.; Mailander, P. C.; Meza, J. L.; Rogan, E. G.; Cavalieri, E. L.; Chakravarti, D. *J. Steroid Biochem. Mol. Biol.* **2008**, *109*, 22–30.
40. Stack, D. E.; Li, G.; Hill, A.; Hoffman, N. *Chem. Res. Toxicol.* **2008**, *21*, 1415–1425.
41. Gaikwad, N. W.; Yang, L.; Muti, P.; Meza, J. L.; Pruthi, S.; Ingle, J. N.; Rogan, E. G.; Cavalieri, E. L. *Int. J. Cancer* **2008**, *122*, 1949–1957.
42. Bocchinfuso, W. P.; Korach, K. S. J. *Mammary Gland Biol. Neoplasia* **1997**, *2*, 323–334.

Downloaded by CORNELL UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch004

43. Bocchinfuso, W. P.; Hively, W. P.; Couse, J. F.; Varmus, H. E.; Korach, K. S. *Cancer Res.* **1999**, *59*, 1869–1876.
44. Devanesan, P.; Santen, R. J.; Bocchinfuso, W. P.; Korach, K. S.; Rogan, E. G.; Cavalieri, E. L. *Carcinogenesis* **2001**, *22*, 1573–1576.
45. Yue, W.; Santen, R. J.; Wang, J. P.; Li, Y.; Verderame, M. F.; Bocchinfuso, W. P.; Korach, K. S.; Devanesan, P.; Todorovic, R.; Rogan, E. G.; Cavalieri, E. L. *J. Steroid Biochem. Mol. Biol.* **2003**, *86*, 477–486.
46. Santen, R. J.; Yue, W.; Bocchinfuso, W.; et al. In *Advances in Endocrine Therapy of Breast Cancer*; Ingle, J. N., Dowsett, M., Eds.; Estradiol-Induced Carcinogenesis via Formation of Genotoxic Metabolites; Marcel Dekker: New York, 2003; pp 13−177.
47. Santen, R.; Cavalieri, E.; Rogan, E.; Russo, J.; Guttenplan, J.; Ingle, J.; Yue, W. *Ann. N. Y. Acad. Sci.* **2009**, *1155*, 132–140.
48. Singh, S.; Chakravarti, D.; Edney, J. A.; Hollins, R. R.; Johnson, P. J.; West, W. W.; Higginbotham, S. M.; Cavalieri, E. L.; Rogan, E. G. *Oncol. Rep.* **2005**, *14*, 1091–1096.
49. Fernandez, S. V.; Russo, I. H.; Russo, J. *Int. J. Cancer* **2006**, *118*, 1862–1888.
50. Gaikwad, N. E.; Yang, L.; Pruthi, S.; Ingle, J. N.; Sandhu, N.; Rogan, E.; Cavalieri, E. *Breast Cancer: Basic Clin. Res.* **2009**, *3*, 1–8.
51. Markushin, Y.; Gaikwad, N.; Zhang, H.; Kapke, P.; Rogan, E. G.; Cavalieri, E. L.; Trock, B. J.; Pavlovich, C.; Jankowiak, R. *Prostate* **2006**, *66*, 1565–1571.
52. Yang, L.; Gaikwad, N.; Meza, J.; Cavalieri, E.; Muti, P.; Trock, B.; Rogan, E. *Prostate* **2009**, *69*, 41–48.
53. Gaikwad, N.; Yang, L.; Weisenburger, D. D.; Vose, J.; Beseler, C.; Rogan, E.; Cavalieri, E. *Biomarkers* **2009**, *14*, 502–512.
54. Pruthi, S.; Yang, L.; Sandhu, N. P.; Ingle, J. N.; Beseler, C. L.; Suman, V. J.; Cavalieri, E. L.; Rogan, E. G. **2011**, submitted.
55. Gail, M. H.; Brinton, L. A.; Byar, D. P.; Corle, D. K.; Green, S. B.; Schairer, C.; Mulvihill, J. J. *J. Natl. Cancer Inst.* **1989**, *81*, 1879–1886.
56. Zahid, M.; Gaikwad, N.; Rogan, E. G.; Cavalieri, E. L. *Chem. Res. Toxicol.* **2007**, *20*, 1947–1953.
57. Samuni, A. M.; Chuang, E. Y.; Krishna, M. C.; Stein, W.; DeGraff, W.; Russo, A.; Mitchell, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5390–5395.
58. Lu, F.; Zahid, M.; Wang, C.; Saeed, M.; Cavalieri, E. L.; Rogan, E. G. *Cancer Prev. Res.* **2008**, *1*, 135–145.
59. Zahid, M.; Gaikwad, N.; Ali, M. F.; Lu, F.; Saeed, M.; Yang, L.; Rogan, E. G.; Cavalieri, E. L. *Free Radical Biol. Med.* **2008**, *45*, 136–145.
60. Zahid, M.; Saeed, M.; Ali, M. F.; Rogan, E. G.; Cavalieri, E. L. *Free Radiacal Biol. Med.* **2010**, *49*, 392–400.
61. Zahid, M.; Saeed, M.; Beseler, C.; Rogan, E. G.; Cavalieri, E. L. *Free Radical Biol. Med.* **2011**, *50*, 78–85.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Chapter 5

Polyphenol Compounds as Antioxidants for Disease Prevention: Reactive Oxygen Species Scavenging, Enzyme Regulation, and Metal Chelation Mechanisms in *E. coli* and Human Cells

Hsiao C. Wang and Julia L. Brumaghim[*]

Chemistry Department, Clemson University,
Clemson, South Carolina 29634-0973
[*]E-mail: brumagh@clemson.edu

Polyphenol antioxidants are abundant in the diet and have the potential to prevent diseases caused by oxidative stress, including neurodegenerative and cardiovascular diseases, cancer, and stroke. Cellular studies are critical to understanding how polyphenol antioxidants can reduce oxidative stress and contribute to disease prevention. Many studies of polyphenol antioxidant mechanisms in *E. coli* and human cells have focused primarily on reactive oxygen species (ROS) scavenging and enzyme regulation, although iron-mediated hydroxyl radical generation and DNA damage is the primary cause of cell death for both prokaryotic and eukaryotic cells under oxidative stress. Recently, metal chelation has also been reported as a major contributor to cellular polyphenol antioxidant activity. Because ROS scavenging, enzyme regulation, and metal chelation mechanisms for polyphenol antioxidant activity are interrelated *in vitro* and in cellular systems, understanding the contribution of each mechanism to polyphenol antioxidant behavior is extremely challenging. Additionally, the wide variety of assays used to examine polyphenol antioxidant behavior makes comparing results between methods and between studies difficult. The complex relationships between ROS scavenging, enzyme regulation, and metal chelation mechanisms make

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

selecting the appropriate cellular experiments vital to accurate mechanistic results, and incorrectly selected methods can lead to conflicting conclusions. Poor understanding of polyphenol cellular uptake also results in inconsistencies between *in vitro,* cellular, and *in vivo* studies. This chapter discusses and compares the evidence to support each of the three major mechanisms for polyphenol antioxidant activity in *E. coli* and human cells and describes the relationships between these three cellular mechanisms.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

1. Introduction

1.1. Polyphenol Antioxidants

Polyphenol compounds are the most abundant and widely studied antioxidants in the human diet. Research concerning the effects of dietary polyphenols on human health has increased considerably in the past twenty years due to worldwide epidemiological studies indicating that consuming foods rich in polyphenols can lower incidence of cancer and chronic heart diseases (*1*, *2*). Polyphenols possess anti-inflammatory, antioxidant, antiallergic, antithrombotic, antiviral, and anti-carcinogenic activities, and polyphenol contributions to antioxidant capacity and beneficial health effects in humans are much larger than that of vitamins (*3*–*6*). An increasing interest in herbal medicines during the past two decades, coupled with an expanded effort to understand the pharmaceutical effects of polyphenols, has made determining cellular mechanisms for polyphenol antioxidant behavior essential to this field.

Oxidative stress and the resulting cellular damage are underlying causes of cancer, neurodegenerative diseases, stroke, and cardiovascular diseases (*7*–*9*). To counteract this oxidative stress, endogenous antioxidants scavenge damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS) (*10*, *11*). Polyphenols are versatile dietary antioxidants that also scavenge ROS and RNS, regulate antioxidant enzyme expression, and chelate metal ions to prevent ROS and RNS formation based on *in vitro* and *in vivo* studies. Growing evidence suggests that these polyphenol antioxidant properties are a major factor in their prevention of cancer, cardiovascular disease, diabetes and aging (*6*, *12*, *13*) in addition to their hypolipidaemic effects, antiviral activity, and anti-mutagenic properties (*14*, *15*).

Polyphenol compounds have attracted recent, intense scientific interest; more than 2000 review papers discussing polyphenol or related compounds have been published since 2001 (*16*). Approximately 1300 of these reviews focus on research progress toward understanding polyphenol antioxidant activity: eighty percent of them discuss possible cellular mechanisms, and the majority of the rest are clinical trial overviews (*17*). Most of the mechanistic review papers focus on polyphenol ROS scavenging or enzyme regulation. A third major mechanism for cellular polyphenol activity, metal chelation, has been examined only in the past several years. Although neglected for many years, a rising interest in polyphenol coordination to biological transition metal ions has

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

brought this mechanism into consideration. In contrast to the ROS scavenging and enzyme regulation mechanisms, cellular metal chelation by polyphenols has not been reviewed in detail, although iron-mediated DNA damage is the primary cause of cell death under oxidative stress for both prokaryotes and eukaryotes (*18–21*). Thus, understanding how polyphenol compounds chelate labile (non-protein-bound) metal ions to reduce cellular oxidative stress is important for a thorough understanding of polyphenol antioxidant function. This review will discuss in detail the metal chelation mechanism for polyphenol antioxidant activity in addition to ROS scavenging and enzyme regulation. Since all three major cellular mechanisms reduce oxidative stress and cell death by reducing ROS and RNS levels, this review will also identify and discuss the relationships between these three polyphenol antioxidant mechanisms and how they might overlap to produce cellular effects.

ROS and RNS scavenging, enzyme regulation, and metal chelation are the three primary cellular mechanisms for polyphenol antioxidant activity in both *E. coli* and human cells. *E. coli* can serve as a simple model for oxidative stress and antioxidant studies; although human cells are more complex than *E. coli*, in some cases, *E. coli* responds to oxidative stress and antioxidant treatment similarly. In addition, the availability of mutant *E. coli* strains with well-understood responses to oxidative stress can help researchers identify cellular mechanisms by which polyphenol compounds prevent oxidative stress. These results can then be extended to and correlated with studies of polyphenol antioxidant behavior in human cells. Understanding cellular polyphenol antioxidant mechanisms may result in predictive models for their antioxidant activity that can be used to identify the most effective antioxidants for animal studies and human clinical trials. This review will also discuss important structural aspects of polyphenol antioxidants to provide general guidelines for the design of multifunctional polyphenols that can exert their cellular effects through multiple antioxidant mechanisms.

1.2. Structures and Sources of Polyphenol Compounds

Polyphenol compounds are commonly found in plants, often as the pigments of leaves, flowers, or fruit. More than 8,000 structurally unique polyphenol compounds have been identified from plant sources, such as fruits, vegetables, nuts, teas, wines, and medicines, but only a limited number are found in milligram quantities per day in most human diets (Table 1) (*22–25*). Polyphenols are a broad class of compounds with different backbone structures and functional groups (Figure 1). The largest and most researched class of dietary polyphenol compounds is the flavonoids, typically the most bioactive and abundant antioxidants found in the diet.

Flavonoids are secondary metabolites in plants and participate in electron transport catalysis during the light-dependent phase of photosynthesis (*26*). Biosynthesis of these compounds starts from glycolysis, which provides the essential substrate phosphoenolpyruvate (PEP; Figure 2). Condensation of PEP with erytheose-4-phosphate (E-4-P) forms 2-keto-3-deoxyarabina-heptulosonate-7-phosphate (DAHP), a product rich in -OH substituents that can be eliminated to introduce double bonds (*27*). Subsequent ring closure and ketone reduction

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

reactions form shikimate, beginning the shikimate acid pathway that converts simple carbohydrate precursors to aromatic amino acids such as tyrosine and phenylalanine (Figure 2) (*27*). Flavonoids are then synthesized from these aromatic amino acids via the phenylpropanoid pathway. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine into the cinnamoyl structure of the flavonoids, followed by conversion to cinnamoyl-CoA by the action of 4-coumaroyl–CoA ligase (4CL). Subsequently, three molecules of malonyl-CoA are condensed with one molecule of cinnamoyl-CoA by chalcone synthase (CHS) to form chalcones (*32*). An isomerization reaction catalyzed by chalcone isomerase (CHI) then stereospecifically converts chalcone to (2*S*)-flavanone, the precursor flavonoid molecule that undergoes alkali-catalyzed condensation to form the different subclasses of plant flavonoids (Figure 1) (*27*, *31*). Flavonoids synthesized by these pathways account for approximately two-thirds of total dietary polyphenol intake (*33*).

The basic flavonoid structure is based on a common three-ring nucleus: two heterocyclic rings fused to an aromatic ring, linked through a heterocyclic pyran or pyrone C ring, normally attached at the C2 or C3 position (Figure 1). Flavonoids are subdivided according to their substituents and are grouped into six subclasses: flavonols, flavones, flavanols, flavanones, isoflavones, and anthocyanidins (Figure 1). Quercetin and myricetin, the main flavonols in U.S. diets, are found in various fruits and vegetables and are especially abundant in onion and cherries (Table 1) (*34*, *35*). Flavones are not as common and can be found in parsley (luteolin) and celery (apigenin) (*35*). Flavanols are found in teas and fruits; the most common class of flavanols are the catechins. Catechins such as epicatechin and epigallocatechin-3-gallate (EGCG) are the main polyphenol compounds in green tea (*28–30*). Polyphenol levels in teas vary considerably depending upon preparation; one study showed that fermenting tea oxidizes catechin compounds and reduces their content by half (*36*). Citrus fruits and juices are the main source of flavanones, especially hesperidin from oranges (*37*, *38*). Isoflavones are found in soy and related products such as tofu; the isoflavones genistein and daidzein have been intensively studied for their estrogenic properties and prevention of breast cancer (*37*, *39*, *40*). Flavonoid substituents affect bioavailability, antioxidant ability, interactions with cell receptors, enzyme regulation, and other biological properties of these molecules.

In addition to substituent differences, dietary flavonoids also exist as polymers or in glycosylated forms, such as flavonoid glycosides that have a sugar molecule bound to the C3 carbon (Figure 1). After consumption, this sugar residue is cleaved to form the biologically active aglycone flavonoids (*41*). The substituents and type of sugar on these flavonoid glycosides greatly determines dietary polyphenol bioavailability, since polyphenol antioxidants must cross cell membranes to exert their biological effects (*41–43*). Hollman and coworkers demonstrated that some glucosides of quercetin were more absorbed in human than pure quercetin, but that quercetin-3-rhamnoglucoside (rutin) was less absorbed (*44*, *45*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 1. Subclasses of flavonoids with individual compounds, food sources, and total measured concentrations

Subclasses	***Compounds***	***Total flavonoid concentrations (mg/100 g)***	***Refs.***
Flavonols	morin, myricetin, quercetin, kaempferol	Red cabbage (25), onion (20-60), broccoli (27), cherries (35-450), cherry tomatoes (1.5-20), apricots (2.5-5), apples (2-24), black grapes (1.5-4)	(*22*, *23*)
Flavones	luteolin, apigenin	Parsley (24-180), celery (2-14), hot peppers (0.5-1)	(*23*)
Flavanols	epicatechin, EGCG, theaflavins	Green tea (4-7), black tea (6-9), chocolate (46-60), grapes (1.5-4), berries (6-27), apples (2-24)	(*24*, *25*)
Flavanones	taxifolin and hesperidin	Citrus fruits and juices (10-140)	(*28*, *29*)
Isoflavones	daidzein	Soybeans (20-90), soy food (60-135), tofu (8-70)	(*29*, *30*)
Anthocyanidins	cyanidine	Red, blue, and purple berries (6-27), red wine (8-30), cherries (35-450)	(*23*)

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

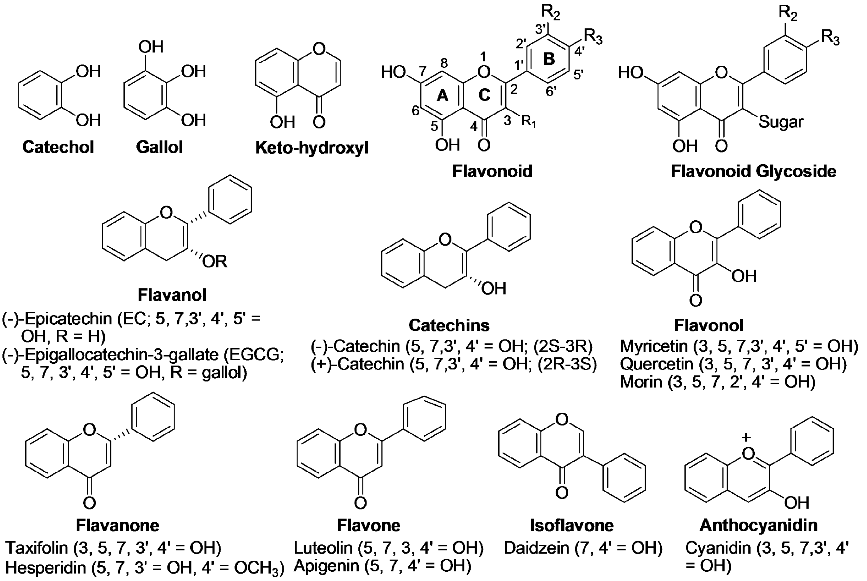

Catechol
Gallol
Keto-hydroxyl
Flavonoid
Flavonoid Glycoside
OH
HO
O
Sugar
R_1
R_2
R_3
A
B
C
1
2
3
4
5
6
7
8
1'
2'
3'
4'
5'
6'
OR
Flavanol
(-)-Epicatechin (EC; 5, 7,3', 4', 5' = OH, R = H)
(-)-Epigallocatechin-3-gallate (EGCG; 5, 7, 3', 4', 5' = OH, R = gallol)
Catechins
(-)-Catechin (5, 7,3', 4' = OH; (2S-3R)
(+)-Catechin (5, 7,3', 4' = OH; (2R-3S)
Flavonol
Myricetin (3, 5, 7,3', 4', 5' = OH)
Quercetin (3, 5, 7, 3', 4' = OH)
Morin (3, 5, 7, 2', 4' = OH)
Flavanone
Taxifolin (3, 5, 7, 3', 4' = OH)
Hesperidin (5, 7, 3' = OH, 4' = OCH_3)
Flavone
Luteolin (5, 7, 3, 4' = OH)
Apigenin (5, 7, 4' = OH)
Isoflavone
Daidzein (7, 4' = OH)
Anthocyanidin
Cyanidin (3, 5, 7,3', 4' = OH)
+

Figure 1. Basic structures of polyphenols and subclasses of flavonoids.

Average daily flavonoid intake in the United States is ~1000 mg (*25*), and concentrations of polyphenol metabolites in human plasma range from 0.33 to 7.6 µM with only 50 to 100 mg flavonoid intake (*44*, *46*, *47*). In murine models supplemented with polyphenols, total plasma polyphenol concentrations may be as high as 1 mM (*48*, *49*), and, in humans, maximum measured plasma concentrations are ~10 µM (*12*, *50*, *51*). In cellular studies of polyphenol supplementation, polyphenol concentrations in *E. coli* and human cells range from 0.1 to 150 µM (*18*, *52–54*). Determining bioavailability and uptake of dietary polyphenols is crucial to determining their biological concentrations and antioxidant effects. Once absorbed, polyphenols are metabolized, and the major metabolic pathways as well as the effects of these processes on polyphenol bioavailability and antioxidant activity are discussed at the end of this chapter. In addition, the concentration of polyphenol compounds in foods varies depending on plant age and processing prior to consumption, complicating animal and clinical studies of dietary polyphenol effects (*55–57*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

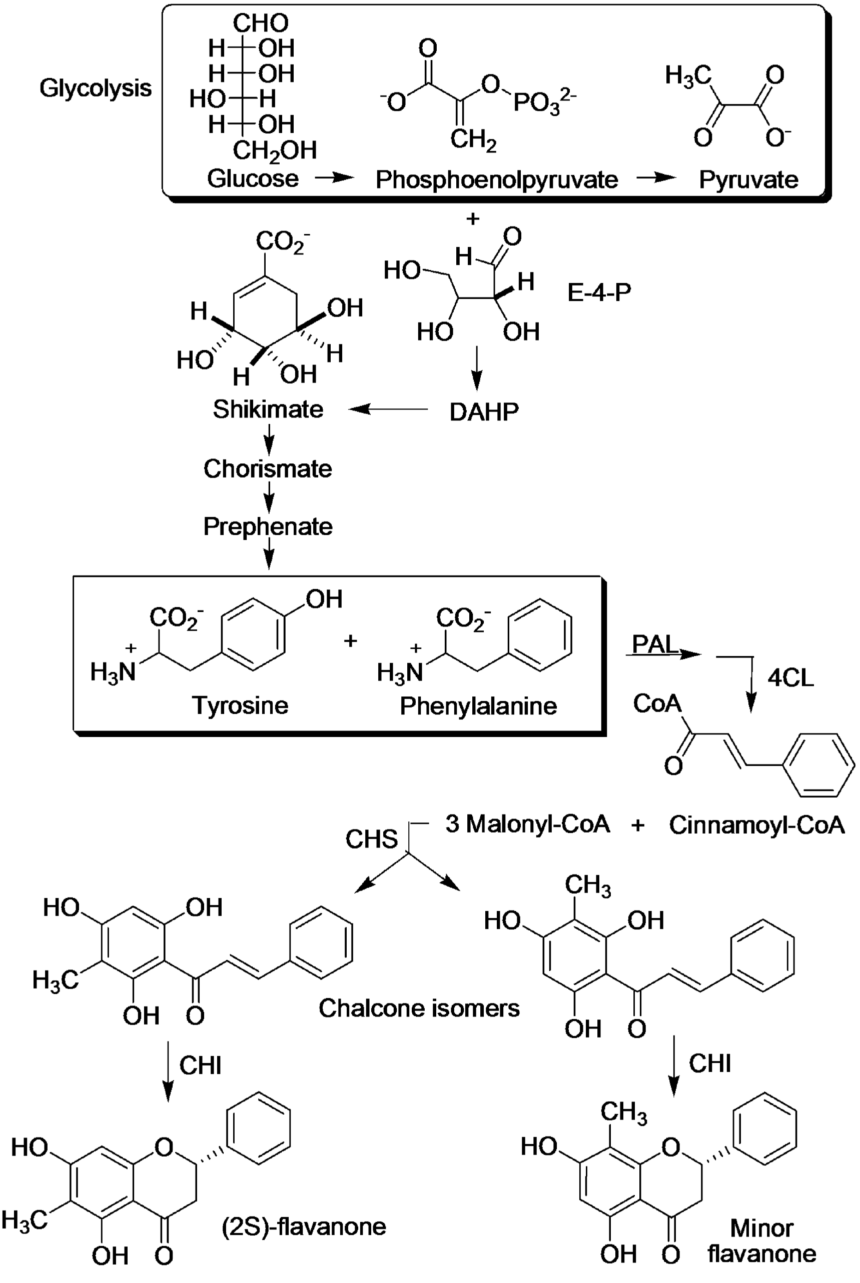

Glycolysis
Glucose
Phosphoenolpyruvate
Pyruvate
+
E-4-P
Shikimate
DAHP
Chorismate
Prephenate
Tyrosine
+
Phenylalanine
PAL
4CL
CoA
CHS
3 Malonyl-CoA + Cinnamoyl-CoA
Chalcone isomers
CHI
CHI
(2S)-flavanone
Minor flavanone

Figure 2. Flavonoid biosynthetic pathways in plants (27, 31, 32).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

2. Polyphenol Antioxidants and Disease Prevention

2.1. Polyphenol Disease Prevention Studies

The American Heart Association and the American Cancer Society both recommend consuming 5 to 9 serving of fruits and vegetables daily because they provide polyphenol antioxidants and other nutrients (*58*, *59*). Since polyphenol compounds are widely available in the diet and are effective antioxidants, many short-term animal and clinical trials focusing primarily on prevention of cardiovascular, Alzheimer's, and Parkinson's diseases as well as cancer have been conducted to establish the effects of various dietary polyphenol subclasses (*2*, *24*, *39*, *60–70*). Table 2 summarizes the animal and clinical studies of polyphenol supplementation for disease prevention in the past decade. The majority of these studies have shown that polyphenol supplementation reduced the oxidative stress that leads to chronic diseases (Table 2), but several studies also determined that high intake of a flavonoid-rich diet did not correlate with cardiovascular disease and cancer prevention (*76*, *78*, *99*, *100*, *104*, *105*).

In addition, studies have shown that mice fed a high fruit and vegetable diet for 5 weeks to 3 months (16-47 mg/d polyphenols) typically have lower risk of cancer, neurodegenerative diseases, and coronary heart disease compared to lower polyphenol intake groups (5-9 mg/d) (*1*, *106*, *107*). A recent review of the short-term (2-6 month) effects of polyphenol rich-food consumption in humans revealed no consistent effect on DNA damage markers or other significant health benefits (*108*). However, many long-duration epidemiological studies (five weeks to two years) indicate that consuming a polyphenol-rich diet decreases the incidence of chronic health problems such as coronary disease, high blood pressure, and cancer (*109–111*). The efficacy and dosage of various polyphenol compounds are still a matter of much debate, although typical dietary polyphenol uptake is around the effective doses (1-50 μM) observed in most cellular and human trials (*50*, *51*, *112*).

Polyphenol antioxidants are thought to prevent cancer cell development by suppressing carcinogenic radical formation, scavenging carcinogenic radicals, and upregulating enzyme levels to increase the detoxification of carcinogenic compounds (*113–115*). Most polyphenol compounds appear to be nontoxic to humans and animals (4-400 mg/kg), but several studies have demonstrated proliferation inhibition in carcinoma cell lines (0.1-100 μg/mL) (*116–118*). Green tea polyphenol compounds have demonstrated cancer preventative effects in mouse studies and epidemiological studies have shown lower cancer rates in areas with high tea consumption (*64*, *119*). In a recent clinical trial, Erba *et. al.* (*101*) investigated the effects of green tea consumption (2 cups/day for 42 days) on oxidative damage, cholesterol levels, and antioxidant status. Tea drinkers showed a ~50% reduction in plasma peroxide levels and a 10% decrease in LDL cholesterol levels compared to the control group, indicating that green tea consumption decreases oxidative stress and leads to fewer incidences of cancer and cardiovascular disease (*120*). Several human trials in various countries have also shown significant cancer prevention ability from oral supplementation with polyphenol compounds such as curcumin, resveratrol, kaempferol, quercetin, catechin, and EGCG (Figures 1 and 3, Table 2) (*63*, *114*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 2. Effects of polyphenol antioxidant supplementation in animal studies and clinical trials

Polyphenol	***Research target***	***Dosage and duration***	***Cellular effects***	***Ref.***
Quercetin	Heart, liver, prostate in rats	50-150 mg/kg/d for 10 d	↓ Phospho-mitogen extracellular kinase ½ (MEK½), phospho-MAP kinase (MAPK), lipid oxidation, and myocyte apoptosis	(*71*)
	Acute glaucoma in rats	400 mg/kg/wk for 6 wk	↑ Heat shock protein 72	(*72*)
EGCG, Catechins	Myocardial ischemia-reperfusion injury in rats	10 mg/kg/h during reperfusion	↓ Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)	(*73*)
	Parkinson's disease in rats	100-400 mg/kg for 2 h	↓ Oxidative DNA damage and NF-*κ*B	(*74*)
	Breast carcinoma in mice	30 mg/kg	↓ c-Jun amino terminal kinases (JNK), NF-*κ*B, AP-1, and tumor growth	(*75*)
	Transgenic adenocarcinoma mouse prostate (TRAMP) model	0.06 % in water for 23 wk	↓ Androgen receptor (AR), insulin-like growth factor-1 (IGF-1), phospho-extracellular signal-regulated kinases 1/2 (phospho-ERKs1/2), cyclooxygenase-2 (COX-2), and nitric oxide synthase (iNOS), ↑ Apoptosis at early stage prostate cancer No effect at late stage prostate cancer	(*76*)
	Lung in albino ICR mice	10 mg/kg before LPS injection	↓ Tumor necrosis factor-alpha (TNF-α) and MIP-2, JNK, extracellular signal-regulated kinases (ERK) 1/2, and NF-κB	(*77*)
	Ovaries in Swiss-Webster mice	12.5-50 mg/kg/d for 7d	↓ Ovarian aromatase CYP19 and cytochrome P450 1A No effects on cytochrome P450 3A and catechol *O*-methyltransferase (COMT)	(*78*)

Continued on next page.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 2. (Continued). Effects of polyphenol antioxidant supplementation in animal studies and clinical trials

Polyphenol	***Research target***	***Dosage and duration***	***Cellular effects***	***Ref.***
	Liver in Sprague-Dawley rats	200 mg/kg/wk for 6 wk	↓ Malondialdehyde (MDA), glutathione peroxidase, and catalase	(*79*)
Resveratrol	Brain in Wistar rats	100 mg/kg after trauma	↓ MDA, xanthine oxidase (XO), and nitric oxide (NO) levels ↑Glutathione (GSH)	(*80*)
	Cerebral ischemia in Mongolian gerbils	30 mg/kg after ischemia	↓ Delayed cell death and activation of glial cells	(*81*)
	Cerebral ischemia in Sprague-Dawley rats	10-100 mg/kg for 48 h	Protected hippocampus mitochondria via sirt1-UCO2 pathway	(*82*)
	Parkinson’s disease in Sprague-Dawley rats	10-40 mg/kg for 10 wk	Protected dopaminergic neurons in *substantia nigra* ↓ Cyclooxygenase-2 (COX-2) and TNF-α	(*83*)
	Alzheimer’s disease in Wistar rats	10-20 mg/kg for 25 d	↓ MDA and nitrite levels, restored GSH and acetylcholinesterase activity	(*84*)
	Huntington’s disease in Wistar rats	5-10 mg/kg for 8 d	↓ MDA ↑ Motor and cognitive impairment and GSH levels	(*85*)
Genistein	Transgenic adenocarcinoma mouse prostate (TRAMP) model	250 mg/kg for 28 wk	↓ MAPK/ERK1/2, tumor growth, estrogen receptor alpha (ER-α), and AR	(*86*)
Silymarin	Prostate cancer patients	570 mg/d for 6 mo	↓ Low density lipoproteins (LDL) and cholesterol ↑ Quality of life score	(*87*)
	TRAMP mice	0.1%-1% diet for 20 wk	↓Cyclin-dependent kinases (CdK), insulin-like growth factor (IGF), and prostate cancer incidence ↑ IGF-binding protein	(*88*)

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Polyphenol	***Research target***	***Dosage and duration***	***Cellular effects***	***Ref.***
	Brain in rats	200-400 mg/d for 7 d	↓ Age-related degeneration, lipid peroxidation, and ROS levels	(*89*)
Curcumin	Brain in rats	5-50 mg/kg/d for 10 d	↓ ROS, MDA, and lipid peroxidation	(*90*, *91*)
	Brain in Wistar rats	10-50 mg/kg for 8d	↓ GSH, lipid peroxidation, and nitrite activity	(*92*)
Olive oil	Healthy volunteers	9-20 mg/d for 3 wk	↓ Oxidized LDL	(*93*)
	Healthy males	4-9 mg/d for 3 wk	↓ Plasma oxidative stress markers	(*94*)
	Healthy males	6-15 mg/d for 1 d	↓ Oxidized LDL (dose-dependent)	(*95*)
	Hypercholesterolemic volunteers	3-16 mg/d for 1 d	↓ Oxidative stress ↑ Endothelial-dependent vasodilatory response	(*96*)
	Stable coronary heart disease	1-8 mg/d for 3 wk	↓ Oxidized LDL and lipid peroxide plasma levels	(*97*, *98*)
	Diabetes volunteers	12.5 mg/d for 4 d	No effect	(*99*)
	Healthy volunteers	4-9 mg/d for 3 wk	No effect	(*100*)
Green tea	Healthy volunteers	2 cups/d (~250 mg catechin) for 42 d	↓ Plasma peroxides and oxidative DNA damage	(*101*)
	Pump workers	6 cups/d for 6 mos	↓ Benzene-induced toxicity, GSH, superoxide dismutase (SOD), and catalase ↑ Malondialdehyde	(*102*)

Continued on next page.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 2. (Continued). Effects of polyphenol antioxidant supplementation in animal studies and clinical trials

Polyphenol	*Research target*	*Dosage and duration*	*Cellular effects*	*Ref.*
	Healthy volunteers	6 g/d for 7 d	↑ Ferric reducing antioxidant power (FRAP) and lipid peroxidase	(*103*)
Soybean flour	HRS/J hairless mice	200 μg/d for 3 d	↓ Hydrogen peroxide production	(*42*)

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

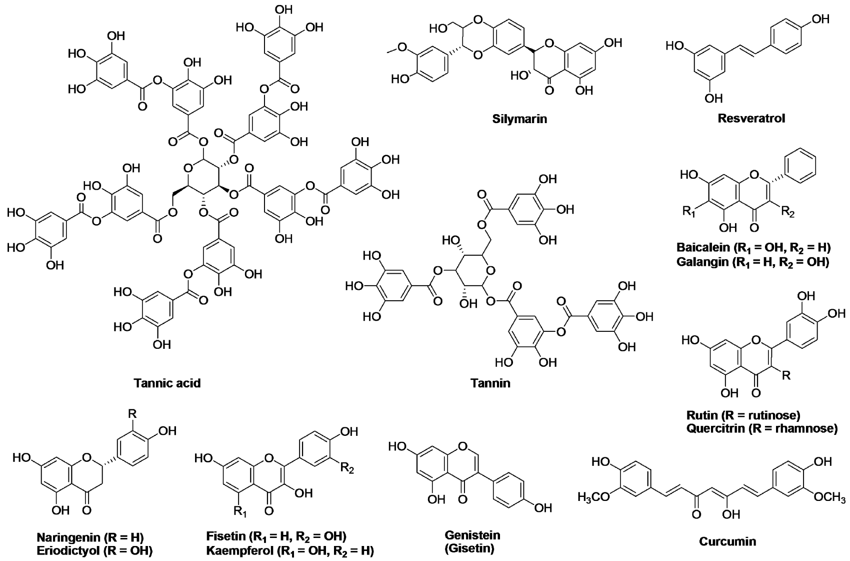

Silymarin
Resveratrol
Baicalein (R_1 = OH, R_2 = H)
Galangin (R_1 = H, R_2 = OH)
Tannic acid
Tannin
Rutin (R = rutinose)
Quercitrin (R = rhamnose)
Naringenin (R = H)
Eriodictyol (R = OH)
Fisetin (R_1 = H, R_2 = OH)
Kaempferol (R_1 = OH, R_2 = H)
Genistein
(Gisetin)
Curcumin

Figure 3. Structures of polyphenol compounds discussed in this review.

In addition to cancer prevention, supplementation studies have also investigated the antioxidant and anti-inflammatory effects of polyphenols on the prevention and treatment of cardiovascular disease (CVD) (*6*, *121–124*). Several studies have reported inverse associations between polyphenol intake and cardiovascular disease incidence (*106*, *120*, *125*, *126*), and Hooper *et al.* (*28*) published a thorough review of the effectiveness of polyphenol subclasses and polyphenol-rich food on CVD and related risk factors (from 133 trials), concluding that a polyphenol-rich diet reduced overall CVD risk factors by lowering LDL levels, decreasing blood pressure, and increasing flow-mediated blood vessel dialatation. In addition, the reduction in CVD incidence due to consumption of olive oil and wine polyphenols has been intensively examined and reviewed in the past decade (*104*, *127–131*). Randomized crossover studies of chronic heart disease patients in several countries showed that consumption of a polyphenol-rich diet decreased oxidative stress, improved the endothelial-dependent vasodilator response, and decreased systolic blood pressure (*97*, *110*, *132–134*).

A four-year follow-up study of green tea drinkers (5910 people who drank 5 or more cups of green tea daily) showed 50 % lower stroke incidence compared a control group consuming less green tea (*135*). Another 15-year study by Keli and coworkers (*136*) also reported a significant reduction in stroke among black tea drinkers and groups with high fruit and vegetable intake. People with high flavonoid intake (28.6 mg/d, mainly quercetin) reduced their risk of stroke by five-fold compared to people with lower flavonoid intake (18.3 mg/d). In addition, higher tea consumption (more than 4.6 cups/day) was found to reduce stroke risk by three-fold compared to lower tea consumption (fewer than2.8 cups/day) (*136*). Polyphenol antioxidants are believed to protect neurons from oxidative

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

damage and death after stroke by ROS scavenging and enzyme regulation (*65*, *83*, *90*, *91*, *137–139*), but recent studies by Weinreb and coworkers have also determined that polyphenol compounds such as EGCG and ECG decrease iron-induced lipid peroxidation and ROS generation by metal chelation (*140*). In contrast, an earlier study by the same group concluded that enzyme regulation was the primary mechanism for polyphenol antioxidant activity based on changes in metal-regulated enzymatic activities (*141*). These results highlight the possibility of overlap between metal chelation and enzyme regulation antioxidant mechanisms that can lead to biased conclusions if multiple mechanisms are not simultaneously examined.

In addition to polyphenol-rich food studies, EGCG and quercetin are the most studied polyphenol compounds in polyphenol supplementation studies for CVD prevention. Male Wistar rats intravenously treated with EGCG (10 mg/kg/h for 2 h) during reperfusion showed reductions in myocardial damage and myeloperoxidase activity (*73*). Plasma IL-6 and creatine phosphokinase levels also decreased after EGCG administration, indicating that EGCG may inhibit nuclear factor-κB (NF-κB) enzyme activity and prevent myocardial damage due to reperfusion. A similar study in Sprague-Dawley rats by Ikizler *et al.* (*142*) showed both short-term and long-term quercetin administration (either 15 mM infusion for 30 min or 50 mg/kg via intragastric tube for seven days) prior to reperfusion-induced oxidative challenge reduced oxidative stress (measured by malondialdehyde, glutathione, glutathione reductase, and nitrite levels) and increased myocardial ischemia recovery by 45% compared to non-treated rats. A review of quercetin and wine polyphenols in animal models and clinical trials by Perez-Vizcaino *et al.* (*143*) also concluded that polyphenols lowered blood pressure and restored impaired endothelial vasodilator function by ROS scavenging in addition to down-regulating NADPH oxidase expression and decreasing ROS production.

Oxidative stress from ROS and RNS can damage nucleic acids, lipids, and proteins in the brain, contributing to neurodegeneneration in Alzheimer's (AD) and Parkinson's diseases (PD) (*140*, *144*). Polyphenol compounds such as EGCG, resveratrol, and curcumin (Figures 1 and 3) have been extensively studied as neuroprotectants to prevent these diseases. Several neuroprotective studies of polyphenol compounds in various models of neurodegenerative diseases *in vitro* and *in vivo* have been published (*126*, *145–148*). A 13-year study with more than 29,000 Finnish subjects by Hu *et al.* showed that coffee (more than 1 cup/day) and tea drinkers (more than 3 cups/day) had 45% and ~60% lower incidences of PD, respectively, compared to those who drink less coffee or tea (*149*). This lowered PD risk was believed to be associated with increased polyphenol intake from these beverages. Karuppagounder and coworkers (*150*) studied the effects of dietary resveratrol in a mouse model of AD by monitoring plaque formation as an indication of AD incidence. Dietary resveratrol supplementation (2% for 45 d) reduced plaque formation by 48-90% compared to mice fed a non-supplemented diet. This diminished plaque formation was linked to decreased glutathione and increased cysteine levels, suggesting that resveratrol reduced oxidative stress by reducing ROS levels (*150*). Although promising results indicate polyphenols may have great potential to treat or prevent neurodegenerative diseases, more mechanistic, cellular, and animal research is required to support clinical trials of polyphenol supplementation.

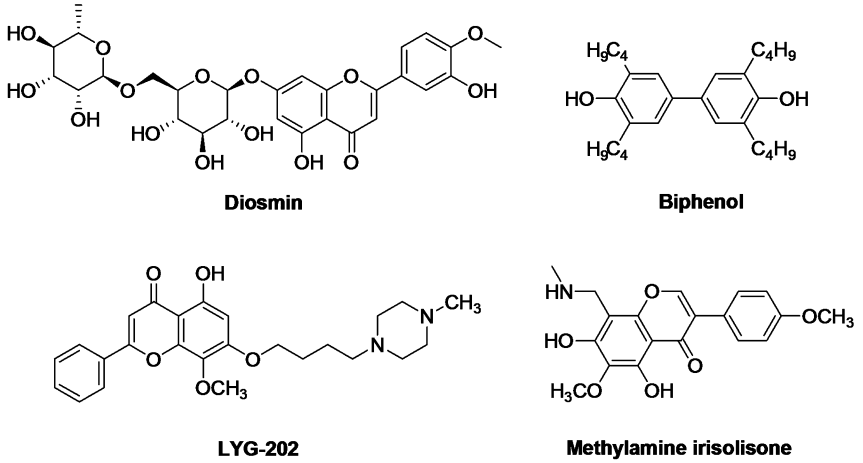
In addition to naturally occurring polyphenol antioxidants, semi-synthetic polyphenol compounds have also been examined for disease treatment (Figure 4) (*69*, *151–154*). Because flavonoid polyphenols can have low water solubilities that may affect bioavailability and antioxidant efficiency (*154–156*), the twin goals of polyphenol modification are to increase water solubility and antioxidant ability. Detralex, a supplement that contains the synthetic polyphenol compound diosmin and the naturally occurring polyphenols hesperidin and rutin (Figure 4), was given orally to 245 patients with varicose vein disease before and after varicose vein removal (1000 mg/day for 14 days before and 30 days after surgery). Diosmin was found to be the active compound, and this treatment reduced pain and postoperative hematomas, possibly due to polyphenol enzyme regulation that accelerated microcirculation (*157*). Rovensky *et al.* (*151*) treated arthritic rats with Detralex (20 mg/kg/d) and methotrexate (0.6 mg/kg/d) for 50 days and measured hind paw swelling, serum albumin and nitrite/nitrate concentrations, and whole-body mineral density to evaluate effects of this treatment on arthritic inflammation. Intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion protein 1 (VCAM1) and other arthritic marker levels were significantly reduced after Detralex treatment, resulting in reduced inflammation and lessening arthritis symptoms. Rovensky and coworkers suggested that increased microcirculation due to Detralex treatment may have been the reason for the observed anti-arthritic effects (*151*).

Duong and coworkers (*153*, *158*) treated cultured human neuronal cells (SH-SY5Y) with the synthetic polyphenol, biphenol (Figure 4), before hypoxiareoxygenation injury. Biphenol treatment (50-100 μM for 1 h) decreased neuronal oxidative stress through ROS/RNS scavenging and inhibited gene expression of hypoxia-inducible factor 1-α (HIF1-α), inducible hemoxygenase 1 (HO-1), glucose transporter 1 (Glut1), the oxygen-sensor neuroglobin (Nb), Cu,Zn-superoxide dismutase (SOD1), catalase (CAT), and glutathione peroxidase 1 (GPx1), increasing cell viability (*153*, *158*). Biphenol was not as potent as other naturally occurring polyphenol compounds such as EGCG and quercetin, and the observed effects of biphenol are at somewhat higher concentrations than the physiologically relevant range of polyphenol concentrations in human plasma (~10 μM) (*51*, *112*, *159*).

Methylamine irisolidone (Figure 4), a synthetic isoflavone derivative with greater water solubility than naturally occurring flavones, reduced myocardial infarction damage in beagles after intravenous injection (40 or 80 mg/kg) 4 h prior to hypoxia-reoxygenation injury (*160*). Mu *et al.* (*154*) also demonstrated that methylamine irisolidone (80 mg/kg) significantly protected against acute myocardial ischemia in beagles by decreasing blood serum lactate dehydrogenase (LDH) and creatine kinase MB (CK-MB) activity, likely by increasing myocardial blood flow and reducing myocardial oxygen consumption.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005



Diosmin

Biphenol

LYG-202

Methylamine irisolisone

Figure 4. Chemical structures of synthetic polyphenols.

The newly synthesized flavonoid LYG-202 (Figure 4) is reported to be a potent antioxidant with anti-cancer and anti-angiogenic effects both *in vitro* and *in vivo* (*69*). Human umbilical vein endothelial cells (HUVEC) pretreated with LYG-202 (0.1-10 μM for 60 min) showed that LYG-202 inhibited angiogenesis and vascular endothelial growth factor (VEGF) signal transduction with an IC_{50} value of ~1 μM (*69*). Although the results of this LYG-202 study were promising, its water solubility, bioavailability, and effects on cellular oxidative stress have not be examined.

Current evidence indicates that polyphenol antioxidants generally protect against diseases caused by oxidative stress; however, evaluating whether individual polyphenol compounds or their combinations have beneficial health effects for groups with specific disease risks remains a challenge in this field. This difficulty is compounded by the fact that results of studies examining polyphenol antioxidant abilities are not always consistent. For example, similar studies by two different research groups to determine the effects of quercetin supplementation on maximal aerobic capacity in humans reported disparate results: one found a positive correlation between quercetin consumption and maximal aerobic capacity, whereas the other observed no correlation (*161*, *162*). Uncertain bioavailabilities and cellular concentrations of polyphenol antioxidants are major issues in these studies, a problem that is compounded by different methods of administration in animal and human trials. Polyphenol hydrophobicity may hinder calculations of effective dosages for *in vivo* studies, and plasma or cellular polyphenol levels are often not measured (*44*, *45*, *163*, *164*). Thus, additional research is needed to establish the effects of polyphenol properties on bioavailability and cellular uptake.

Human and animal trials with polyphenol antioxidants have increased significantly over the past decade, but the variety of polyphenols used for these

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

trials, mixed outcomes, and unclear cellular mechanisms make it difficult to establish accurate biological effects for different polyphenol subclasses (*3*, *37*, *55*, *73*, *80*, *94*, *95*, *161*, *165*, *166*). Most clinical trials with polyphenol compounds focus on oxidative stress inhibition through ROS scavenging and enzyme regulation (*55*, *95*, *167*); few consider the possibility of metal chelation effects. Lack of understanding the cellular mechanisms responsible for the results of these studies, or focusing only on a single mechanism for polyphenol activity, may result in under- or overestimating the importance of a particular mechanism, leading to incorrect or conflicting conclusions and slowing progress in this field (*107*, *146*, *168–170*). Since all three mechanisms lead to oxidative stress reduction, possible overlap between ROS scavenging, enzyme regulation, and metal chelation mechanisms should be carefully considered in future studies. Understanding and examining the interrelationships between the three major mechanisms for polyphenol antioxidant activity in cells will provide a better foundation for understanding the results of polyphenol animal and clinical trials and enable the development of polyphenols that act through multiple mechanisms to prevent disease.

2.2. Oxidative Stress and Cellular Effects

Oxidative stress is a primary cause of DNA damage and an underlying cause of many chronic diseases, including cardiovascular disease, cancer, and neurodegenerative diseases (*59*, *113*, *145*, *147*, *171*, *172*). Both ROS and RNS are sources of oxidative stress; therefore, preventing formation or scavenging cellular ROS and RNS is believed to be a major mechanism for polyphenol antioxidant activity (*9*, *10*). Many sources of ROS and RNS are present in cellular environments (Figure 5): 1) metal-mediated reactions such as the Fenton reaction (*19*); 2) pollutants and toxins (*173*); 3) irradiation by UV light, X-rays, or gamma-rays (*8*); 4) microphage-produced ROS and RNS during inflammation (*174*, *175*); and 5) side products of mitochondria-catalyzed electron transport reactions (*176–178*). ROS and RNS play both beneficial and deleterious roles in cells. At low concentrations, ROS and RNS play a major role in intracellular signaling and enzyme regulation (*179*). Superoxide radical ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) also induce mitogenic responses to defend against infectious agents (*173*). In contrast, high ROS and RNS concentrations resulting from imbalances in reactive species formation and antioxidant defenses lead to uncontrolled increases in the steady-state concentrations of these oxidants and radical-mediated chain reactions that cause DNA lesions, lipid peroxidation, and protein damage (*10*, *11*, *180*). Under oxidative stress conditions, high concentrations of ROS and RNS can cause cell death either via apoptosis or necrosis (*66*).

Mitochondrial and nuclear DNA are both major targets for ROS and RNS damage, including base modification, double-strand breakage, and DNA-protein cross-links (*10*, *181*). Although histone proteins are thought to protect nuclear DNA from this damage, several studies showed that metal-mediated DNA damage *increases* in the presence of histone proteins (*182–184*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

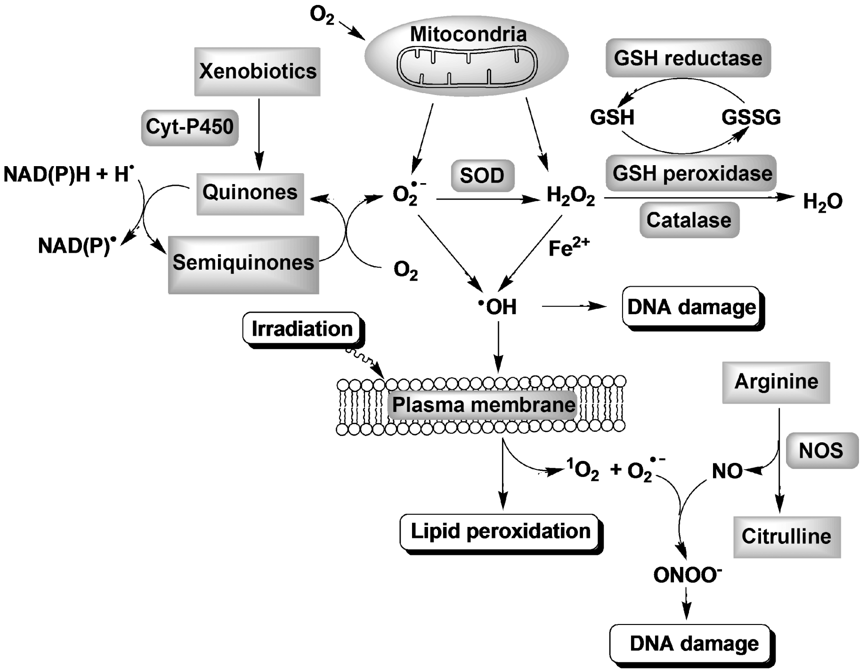

O2
Mitocondria
Xenobiotics
Cyt-P450
GSH reductase
GSH
GSSG
NAD(P)H + H•
NAD(P)•
Quinones
Semiquinones
O2•-
SOD
H2O2
GSH peroxidase
Catalase
H2O
O2
Fe2+
•OH
DNA damage
Irradiation
Plasma membrane
Arginine
NOS
NO
1O2 + O2•-
Lipid peroxidation
Citrulline
ONOO-
DNA damage

Figure 5. Scheme showing the major cellular sources of oxidative stress.

Oxidative DNA damage contributes to the development of a wide range of diseases including Alzheimer's disease (*185*), Parkinson's disease (*66*, *138*), diabetic pathologies (*13*), cardiovascular diseases (*12*, *186*), rheumatoid arthritis (*10*, *11*), cancer (*5–7*), and neurodegeneration (*138*). ROS and RNS react with DNA at diffusion-controlled rates (~2-10 × 10^9 $M^{-1}s^{-1}$) by addition to double bonds in DNA bases or by abstracting a hydrogen atom from the deoxyribose sugar or the methyl group of thymine (*187*). Although cellular mechanisms can repair this damage, significant amounts of damaged DNA can effect replication and transcription, leading to mutagenesis (*18*, *188*, *189*). Thus, damaged DNA nucleotides such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) are established biomarkers for oxidative stress as well as the potential mutagens, and 8-oxo-dG levels are used as intermediate markers for diseases such as cancer (*190*, *191*).

Cellular enzymatic and non-enzymatic antioxidant defenses are crucial to preventing ROS and RNS toxicity, cellular damage, and oxidative stress. Antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione-regenerating enzyme systems (*192*, *193*). SOD and catalase decompose ROS and RNS directly, whereas other enzyme systems reduce oxidized thiols (*62*, *174*). Non-enzymatic antioxidant defenses are less specific, but ROS/RNS scavenging and metal chelation by small-molecule antioxidants can reduce or inhibit ROS/RNS generation (*194*). Several well-known biomarkers are used to measure the extent of cellular oxidative damage by ROS and RNS, as well as polyphenol prevention of this damage, including peroxidation of membrane lipids, oxidative damage to nucleic acids and carbohydrates, and protein oxidation (*7*, *148*, *195*).

2.3. Reactive Oxygen and Nitrogen Species Formation

Superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are generated primarily in the mitochondria by cytochrome P450 (Cyt-P450) metabolism, in peroxisomes, and by inflammatory responses (*179*, *196*). Under physiological conditions, mitochondria are responsible for ~2 % of total oxygen uptake in an organism and are the primary generators of H_2O_2 (*196*). In addition, during energy transport through the electron transport chain, ~3% of electrons are transferred to O_2 in the mitochondria, making mitochondria a significant source of cellular $O_2^{\bullet-}$ (*181*). Superoxide anion can further react to generate secondary ROS, either directly or through enzyme or metal-mediated reactions (Figure 6) (*175*). Since the majority of ROS are produced in mitochondria (*113*), to minimize the possibility of ROS imbalance, mitochondria also contain high concentrations of small-molecule antioxidants and antioxidant enzymes on both sides of the membrane, primarily superoxide dismutase superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx) (*175*). Outside mitochondria, xanthine oxidase (XO) and Cyt-P450 can also reduce oxygen to generate superoxide and H_2O_2 from the reaction of xanthine to uric acid or the Cyt-P450 catalytic cycle, respectively (*189*). Other possible endogenous sources of ROS are the oxidation of fatty acids in the peroxisome, NADPH oxidase, and the tricarboxylic acid (TCA) cycle enzyme, but these processes generate only ~3% of cellular ROS compared to mitochondria (*197*).

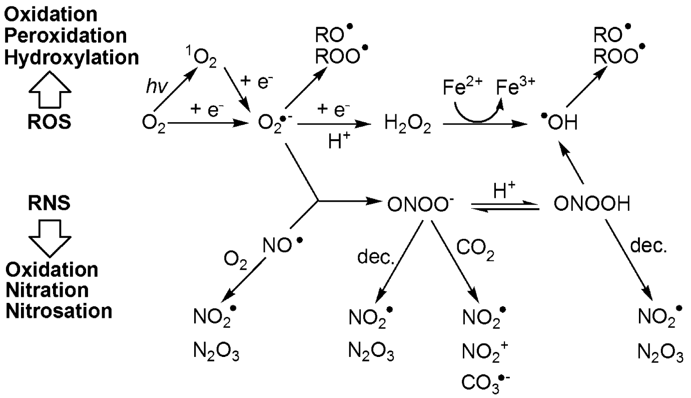


Figure 6. Reactions that generate reactive oxygen species (ROS) and reactive nitrogen species (RNS).

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

In addition to $O_2^{\bullet -}$ and H_2O_2, hydroxyl radical ($^{\bullet}OH$) is also a highly reactive and damaging species with a half-life *in vivo* of approximately 10^{-9} s (*198*). The reaction between Fe^{2+} and H_2O_2 is often referred to as the Fenton reaction (Reaction 1), and has a rate constant of $2 \times 10^4\ M^{-1}s^{-1}$ (*199*). The Fenton reaction generates $^{\bullet}OH$ that reacts with either DNA bases or sugars to cause base oxidation or DNA strand breakage as well as other types of cellular damage (Figure 6) (*18*, *19*, *29*, *66*, *179*, *198*, *200*, *201*). Production of hydroxyl radical is primarily a metal-mediated process catalyzed by Fe^{2+} and Cu^+ (*18*, *66*, *202*), although other redox-active metal ions, such as Cr^{3+} and CO^{2+}, are also strong candidates for cellular generation of $^{\bullet}OH$ from H_2O_2 (*202*, *203*). Metal-mediated $^{\bullet}OH$ generation under physiological conditions is controlled by the concentrations of non-protein-bound metal ions available to react with H_2O_2 and generate $^{\bullet}OH$. Under oxidative stress conditions, excess H_2O_2 and $O_2^{\bullet -}$ can directly oxidize non-protein-bound Fe^{2+} as well Fe^{2+} in iron-sulfur clusters [4Fe-4S] (*179*). Iron and copper overload conditions such as hemochromatosis, β-thalassemia, and Wilsons disease result in increased non-protein-bound Fe^{2+} or Cu^+ levels, resulting in increased oxidative stress (*66*, *70*, *204–207*). Besides endogenous pathways, ROS can also be produced from environmental agents such as carcinogens, chlorinated compounds, toxic metal exposure, radiation, and barbiturates (*8*, *11*, *208*).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (1)$$

Reactive nitrogen species also cause oxidative stress; nitric oxide ($NO^{\bullet}$) is a radical produced by nitric oxide synthase (NOS) enzymes (*174*) with a short half-life (~2 s) under ambient O_2 conditions (*173*, *203*) and a longer half-life (~15 s) under limited oxygen concentrations (*174*). $NO^{\bullet}$ exerts its main physiological and pathological effects by inhibiting cytochrome oxidases and may also regulate mitochondrial antioxidant mechanisms (*174*). During inflammatory processes, cells produce both $O_2^{\bullet -}$ and $NO^{\bullet}$ that can be converted into other reactive species such as nitrosothiols, peroxynitrous acid (HOONO), and hydrogen peroxide (H_2O_2), leading to oxidative stress. Peroxynitrous acid formation from $O_2^{\bullet -}$ and $NO^{\bullet}$ has an extremely high rate constant ($6.2 \times 10^9\ M^{-1}s^{-1}$) (*174*) and its anion, peroxynitrite ($ONOO^-$), is believed to be a major source of oxidative stress. Peroxynitrite has a half-life of ~1 s at pH 7.4 and 37 °C; thus, it will either oxidize proximal target molecules *via* production of secondary reactive species or decompose into non-damaging byproducts (*174*). Peroxynitrite can also directly react with molecules such as carbon dioxide; thiols in iron-sulfur clusters; zinc fingers and other proteins; amines; and both free and bound transition metals (*209*). ROS and RNS generation pathways are complex and varied, often making it difficult to ascertain specific cellular polyphenol scavenging mechanisms.

3. General Polyphenol Antioxidant Mechanisms in Cells

Polyphenols are reported to have several mechanisms for their observed antioxidant activity and great debates have arisen as to which mechanisms are most important in cells. ROS scavenging and cell signaling regulation mechanisms have been the major focus in the past two decades, but there has been rising interest in metal chelation and other mechanisms, such as calcium channel activation, in recent years (*29*, *210–212*). Polyphenol compounds have been identified as potent antioxidants due to their ROS and RNS scavenging abilities, strong iron and copper binding properties, and strong induction of endogenous antioxidant enzymes, in particular xanthine oxidase, NADPH oxidase, and lipoxygenase (*4*, *107*, *213–217*). Besides oxidative stress inhibition, polyphenols also prevent expression of inflammatory signaling molecules, platelet aggregation, and vascular constriction (*62*, *66*, *147*, *218*). Understanding the contributions from each of these cellular mechanisms for polyphenol antioxidant behavior will help researchers identify and design the most effective and least toxic polyphenol antioxidants. In this chapter, the ROS and RNS scavenging, enzyme regulation, and metal binding mechanisms for polyphenol antioxidant activity will be closely examined; these are the three most commonly studied mechanisms, and each has significant scientific support. Since metal chelation has not been widely studied as an antioxidant mechanism until recently, this chapter will focus on describing studies related to polyphenol-metal chelation in cells and highlighting the correlations between all three mechanisms that must be addressed when determining primary modes of cellular polyphenol antioxidant activity.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

3.1. ROS and RNS Scavenging Mechanisms

Polyphenols can neutralize cellular ROS and RNS to prevent further radical reactions and damage. Under cellular oxidative stress conditions induced by hypoxia, excessive prooxidant concentrations, or treatment with chemotherapeutic drugs such as adriamycin, polyphenol compounds can reduce DNA and other cellular damage by scavenging radicals (*127*, *219*, *220*). Polyphenol radical scavenging assays are generally performed in aqueous solution using various methods, including the Trolox-equivalent antioxidant capacity assay (TEAC), the ferric reducing ability assay (FRAP), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, inhibition of red blood cell hemolysis induced by peroxyl radical, and the inhibition of Cu^{2+}-induced plasma oxidation (*221–223*). Because these methods are often quite sensitive to small changes in experimental conditions, results can vary even when testing the same polyphenol using the same methods.

Since naturally occurring polyphenol antioxidants can be hydrophobic at physiological pH, their radical scavenging abilities in lipophilic environments are also of interest. For example, polyphenols have been reported to interfere with lipid oxidation by donating a hydrogen atom to lipid-oxidizing hydroxyl radical ($^{\bullet}OH$) to form a stable phenoxy radical intermediate ($PhenO^{\bullet}$), incapable of further radical reaction (Reaction 2) (*224*). Unfortunately, more complicated procedures must be used to perform radical scavenging studies in lipophilic environments, causing additional sources of variation in experimental results (*152*). In addition, antioxidant assays often have different standards by which they rate antioxidant capacity; thus, it is often difficult to directly compare results from different ROS or RNS scavenging assays (*225*). Another key difficulty is the inconsistency between *in vitro* and cellular studies; since polyphenol compounds must cross cell membranes to exert their antioxidant effects inside cells, cellular polyphenol antioxidant studies often lower efficacy compared to similar *in vitro* experiments (*226*, *227*).

$$\text{Polyphenol (PhenOH)} + {}^{\bullet}\text{OH} \rightarrow H_2O + \text{PhenO}^{\bullet} \qquad (2)$$

In general, polyphenol compounds have shown greater ROS scavenging abilities with increasing numbers of hydroxyl substituents, but the positions of these hydroxyl groups are also critical (*11*). Catechol and gallol groups, with at least two adjacent hydroxyl groups on the B ring (Figure 1), have been determined to be the most important substituents for increasing ROS scavenging ability. Theoretical calculations and *in vitro* experiments to determine polyphenol antioxidant efficacy indicated that removal of one hydroxyl group from a catechol moiety results in a marked decrease in radical scavenging ability (*43*). Similar *in vitro* studies have shown that adding an additional hydroxyl group on the B ring, to form a gallol moiety, increased radical scavenging ability compared to catechol analogs, but addition of a third hydroxyl group did not result in as significant a change in ROS scavenging ability as addition of a second hydroxyl group (*43*, *228*). In addition, the presence of a C2-C3 double bond on the C ring increased scavenging activity, likely because it stabilizes the phenoxy radical intermediate after reaction with a radical species. Presence of a 4-oxo substituent (a C=O double bond at the C4 position) also increased scavenging activity by delocalizing electrons from the B ring (*170*, *212*, *228*). Additional hydroxyl groups on the C5 and C7 positions also increased radical scavenging potential, but these hydroxyl substituents must be accompanied by a C2-C3 double bond on the C ring (*108*, *228*).

Quercetin and resveratrol (25-100 mg/kg) decreased lipid peroxidation in human plasma and blood platelets (*164*, *229*); similar effects were also observed in murine studies (*230*). The most abundant polyphenol from green tea, EGCG, was found to have strong radical scavenging abilities and also inhibited lipid peroxidation (*107*, *231*). Polyphenols extracted from olive oil also were reported to have strong ROS scavenging abilities (*94*, *232*). Faine and coworkers compared the antioxidant capacities of olive oil (7.5 mL/kg/d) and its isolated components, oleic acid (3.45 ml/kg/d) and the polyphenol dihydroxyphenolethanol (7.5 mg/kg/d), in male Wistar rats that received gavage treatments twice a week for 30 days (*232*). The olive-oil-treated rats showed a 40% decrease in atherogenesis risk compared to the control group. In addition, olive oil was more effective than its isolated components, decreasing both total superoxide dismutase (SOD) activity and oxidized protein carbonyl concentration by ~30% (*232*). A separate study by Visavadiya *et al.* determined the $O_2^{\bullet-}$, ${}^{\bullet}$OH, and NO$^{\bullet}$ scavenging abilities of polyphenol-rich sesame plant extracts using DPPH, TEAC, and lipid peroxidation assays (*169*). These *in vitro* assays demonstrated that sesame-derived polyphenols scavenged radicals with 50% inhibition concentrations (EC_{50} values) of 1-35 μM, similar to the standard radical-scavenging agents mannitol, α-tocopherol, and Trolox (*169*), and consistent with the beneficial effects of these extracts observed in mammalian cell studies (3.4-100 μM) (*62*, *114*, *232*–*234*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

3.2. Enzyme Regulation Mechanisms

Regulation of enzymatic activity is another widely-studied cellular mechanism for polyphenol antioxidant activity, and several reviews have detailed the effects of polyphenol compounds, mainly flavonoids, on enzyme regulation in both *E. coli* and human cell studies (*11*, *127*, *138*, *235*). Many *in vitro* studies of polyphenol enzyme regulation have also been performed (*210*, *236–240*). Celik *et al.* (*233*) used gel electrophoresis to determine *in vitro* DNA damage inhibition by quercetin, rutin, naringenin, and resveratrol (Figure 3) on idarubicin- and mitomycin-induced oxidative stress. Quercetin was the most potent antioxidant, inhibiting 50% DNA damage (IC_{50}) at ~50 μM; the other tested polyphenol compounds had IC_{50} values in the millimolar range. Trolox, a water soluble ROS scavenger, did not show protective effects in this study, indicating that ROS scavenging ability was not primarily responsible for the observed effects. Thus, the authors concluded that these polyphenol antioxidants inhibited ROS generation and DNA damage by interacting with cytochrome P450 (*233*).

Animal studies have also provided suggestive results that polyphenol compounds regulate enzyme function. Visavadiya *et al.* (*241*) demonstrated that supplementation with a polyphenol-rich sesame diet for four weeks improved LDL level, HDL level, and atherogenic index in hypercholesteraemic rats by ~30%. Both catalase and SOD levels increased by 15-25%, indicating that polyphenols might increase activities of these enzymes to reduce lipid peroxidation by 23% (*241*). A separate study by Qian *et al.* measured the antioxidant activity of luteolin used in male Sprague-Dawley rats with high-glucose-induced diabetes (*242*). Overall, diabetic rats showed higher levels of $^{\bullet}OH$ and ROS formation and lower SOD and NOS activities compared to the control group. Luteolin-treated rats (oral administration of 100 mg/kg for 8 weeks) had significantly lower $NO^{\bullet}$ and $^{\bullet}OH$ levels and increased SOD and NOS expression, whereas treatment with the established ROS scavenger mannitol showed no significant effects. In addition, luteolin treatment for eight weeks (10, 50, and 100 mg/kg/d) promoted endothelium-dependent relaxation in diabetic rats, an effect that was inhibited by pretreatment with the NOS inhibitor L-NAME (L-N^G-nitroarginine methyl ester), but not by indomethacin, a cyclooxygenase inhibitor. These results indicated that luteolin lessened oxidative stress by reducing ROS generation and enhancing NOS activity (*242*).

Polyphenol antioxidants have strong protein-binding abilities, and alterations of protein conformation upon binding can lead to changes in enzyme activity and expression (*115*, *243*). However, concentrations of polyphenol compounds required to affect enzyme regulation are often higher than those required for ROS scavenging and metal chelation, so the general cellular relevance of this mechanism remains uncertain. In some cases, it may not be possible to identify enzymatic regulation effects, due to the lack of suitable biomarkers, or to distinguish direct enzymatic effects from upstream regulatory effects, since many cellular pathways may be involved in single-enzyme regulation. In addition, polyphenol ROS scavenging or metal chelation antioxidant mechanisms can overlap with enzyme regulation (e.g., polyphenol reduction in ROS levels due to scavenging may alter levels of antioxidant defense enzymes), leading to

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

incorrect conclusions if multiple mechanisms for antioxidant activity are not simultaneously examined.

3.3. Metal Chelation Mechanisms

Redox-active metal ions, primarily Fe^{2+} and Cu^{+} because they are most abundant, can generate ROS and RNS in cells (*244*). In fact, iron-mediated DNA damage is the primary cause of cell death under oxidative stress conditions in both *E. coli* and human cells (*18*, *19*, *176*, *203*, *206*). Polyphenol compounds have pK_a values in the range of 6-9 (*29*), near physiological pH, and are therefore partially deprotonated *in vivo*. Iron can bind polyphenols in a bidentate fashion through two oxygen atoms of deprotonated catechol and gallol groups (Figure 7), and this binding can prevent ROS generation (*29*). Polyphenol compounds strongly bind iron: the stability constant (K_f) of the Fe^{2+} monocatecholate complex is $10^{7.9}$ (*29*), compared to the Fe^{3+} monocatecholate complex with a $K_f \cong 10^{20}$ (*29*), indicating that formation of Fe^{3+}-polyphenol complexes are thermodynamically favored over Fe^{2+} complexes. Thus, once bound, Fe^{2+}-polyphenol complexes undergo spontaneous autoxidation in the presence of O_2 to form Fe^{3+}-polyphenol complexes (*214*, *245*).

Perron *et al.* used gel electrophoresis methods to establish that polyphenol antioxidants inhibit *in vitro* iron-mediated DNA damage with IC_{50} values ranging from 1-59 μM (*156*). Additional DNA damage assays and UV-vis experiments further confirmed that iron binding was essential for the observed polyphenol antioxidant activity. Inhibition of iron-mediated DNA damage was found to strongly correlate with both polyphenol pK_a values (*156*) as well as the rate of polyphenol-Fe^{2+} autoxidation (*245*). Both correlations are expected if polyphenol-iron chelation is a primary antioxidant mechanism, since polyphenol-iron binding and stabilization of Fe^{3+} will inhibit $^{\bullet}OH$ generation (Reaction 1) (*156*).

Visavadiya *et al.* (*169*, *241*) used the Fenton reaction to generate $^{\bullet}OH$ radical and compared 2-deoxyribose damage prevention with and without addition of polyphenol-rich sesame extracts. Addition of the polyphenol-rich extracts reduced iron-mediated 2-deoxyribose damage with IC_{50} values of 50-100 μM, and addition of the iron chelator ethylenediaminetetraacetic acid (EDTA) prevented the observed antioxidant activity. Although the polyphenol extracts prevented *in vitro* 2-deoxyribose damage by iron binding at lower concentrations than by ROS scavenging, similar mammalian cell studies showed a closer correlation to *in vitro* ROS scavenging (measured by DPPH, FRAP, and H_2O_2/Fe^{2+} assays) relative to polyphenol iron-binding ability (measured by UV-vis spectroscopy), leading the authors to conclude that sesame polyphenols inhibited DNA damage and increased cell survival primarily by ROS scavenging (*169*, *241*). However, H_2O_2/Fe^{2+} ROS scavenging assay results correlated more strongly with sesame extract prevention of cell death than the DPPH and FRAP assay results, so it is not clear whether iron binding and ROS scavenging mechanisms were sufficiently distinguished in this study.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

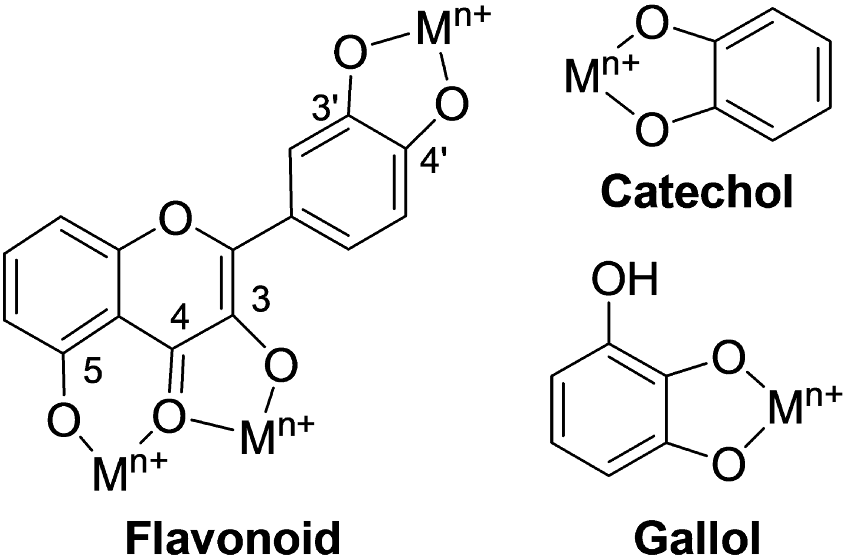


Figure 7. Potential binding sites for polyphenol compounds.

Kim and coworkers (*200*) compared the ROS scavenging and iron chelation capacity of Pycnogenol, an extract from French maritime pine bark, and found that Pycnogenol and curcumin reduced DNA damage by inhibiting the Fenton reaction instead of ROS scavenging. Pycnogenol (50 µg/mL) inhibited iron-mediated DNA damage by 88% if added before the Fenton reaction was initiated by H_2O_2 addition, but Pycnogenol showed little or no effect on DNA damage after the Fenton reaction was initiated. These results suggested that Pycnogenol antioxidant activity resulted from iron chelation inhibiting $^{\bullet}$OH generation, not from direct $^{\bullet}$OH scavenging (*200*). Mandel *et al.* (*141*) showed that the gallol-containing polyphenol EGCG exerts its neuroprotective effects in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice through iron binding to the gallol groups. This iron binding was sufficient to reduce labile iron levels by ~38%, an effect comparable to that of the established VK-28 series of iron chelators (*141*). Mira *et al.* and Fernandez *et al.* used spectrophotometry methods to compare the metal chelating abilities of twelve polyphenol compounds, and determined that polyphenol compounds with catechol or gallol groups were the most effective Fe^{2+}, Fe^{3+}, Cu^{+}, and Cu^{2+} chelators (*156*, *244*, *246*, *247*).

In addition to catechol and gallol groups, polyphenols such as quercetin and myricetin also have two additional sites that can bind transition metals: between the 5-hydroxyl and 4-carbonyl groups (5-hydroxyl-4-keto site) or between the 3-hydroxyl and 4-carbonyl groups (3-hydroxyl-4-keto site) of the C ring (Figure 6). Compared to catechin, myricetin has additional metal binding sites at the 5-hydroxyl-4-keto and 3-hydroxyl-4-keto sites and more effectively chelated both Fe^{2+} and Cu^{+} (*247*). Studies of metal binding to rutin and quercetin showed that at physiological pH, metal binding at the 3-hydroxyl-4-keto site was favorable compared to metal binding at the 5-hydroxyl-4-keto site, most likely due to

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

pK_a differences (*244*)(*247*). Unsurprisingly, polyphenol methylation at the 3-hydroxyl site significantly decreased antioxidant activity by blocking a metal binding site, whereas methylation of the 7-hydroxyl site lowered the pK_a of the 3-hydroxyl-4-keto site, increasing antioxidant activity (*248*). Removing the C3-hydroxyl substituent in luteolin to form kaempferol resulted in a remarkable decrease in both general metal chelating ability and antioxidant ability (*247*, *249*). Polyphenols can also inhibit copper-mediated oxidative stress by binding copper (*203*). In fact, flavanones without catechol or gallol groups, such as naringenin and naringin, were found to bind Cu^+ but not Fe^{2+} (*247*, *250*). Since polyphenol compounds have pK_a values ranging from 6 to 9 (*29*, *156*), their partial ionization at physiological pH will affect both cellular bioavailability and metal binding. Thus, polyphenol bioavailability and metal binding ability should be considered together when estimating the potency of polyphenol antioxidants in cells.

In some cases, polyphenol compounds may also act as prooxidants. Polyphenol compounds with catechol groups are reported to promote oxidative DNA damage by $Fe(EDTA)^-$, due to high polyphenol reduction potentials that can reduce Fe^{3+} to Fe^{2+} and promote the Fenton reaction (*169*, *251*). Previous studies have shown that polyphenols reduced iron in the Fe^{3+}-bleomycin complex to form Fe^{2+} and promote iron-mediated DNA damage (*252*). In addition to iron, copper also produces hydroxyl radical in a Fenton-like reaction, resulting in DNA and cell death, and polyphenol compounds have demonstrated both antioxidant and prooxidant effects in the presence of copper (*29*, *202*, *207*, *253*, *254*). Since both antioxidant and prooxidant effects of polyphenol compounds have been observed, depending on both the metal and the polyphenol, it is vital to understand how these individual compounds behave in cellular environments.

Polyphenol antioxidant research and reviews have primarily focused on direct ROS and RNS scavenging and other possible mechanisms, including enzyme regulation and metal chelation, have not received as much attention. Because single studies examining all three polyphenol antioxidant mechanisms are rare, these antioxidant mechanisms can seem very separate. Although ROS/RNS scavenging is the most widely examined polyphenol antioxidant mechanism, to overlook enzyme regulation or metal chelation mechanisms may underestimate polyphenol antioxidant abilities (*244*).

Inhibition of oxidative stress by polyphenol-metal chelation has been studied only recently, but this mechanism has the potential to significantly reduce oxidative damage because metal chelation can inhibit ROS formation rather than scavenge ROS after they form. Studies have shown that polyphenol antioxidants inhibited DNA damage with IC_{50} values lower than ROS scavenging and enzyme regulation mechanisms, indicating that metal chelation is a major factor in polyphenol antioxidant behavior (*122*, *255–258*). In addition, several studies also used iron-generated $^{\bullet}OH$ radical to determine polyphenol $^{\bullet}OH$ scavenging ability (*68*, *169*, *200*, *259*), potentially confounding ROS scavenging and metal chelation mechanisms. In the following sections, all three polyphenol antioxidant mechanisms will be examined and compared for both *E. coli* and human cells to provide a more complete understanding of cellular polyphenol antioxidant behavior.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

4. Oxidative Stress and Antioxidant Defenses in *Escherichia coli*

E. coli is a thoroughly researched organism often used in studies to test simpler cellular systems. A primary advantage of *E. coli* is its ability to grow very quickly compared to mammalian cells (30 min/generation under optimal conditions), and the ease with which it can be manipulated, both genetically and biochemically (*260*). In addition to its simplicity, safety, and well-established genetic properties, another major asset of *E. coli* is its ability to take up exogenous genetic material by DNA-mediated cell transformation, making it a popular model for recombinant DNA studies (*261*). Testing polyphenol effects on mutant *E. coli* strains, such as those defective in DNA repair or iron uptake regulation, can provide a better understanding of cellular polyphenol antioxidant mechanisms (*179*, *262–265*).

4.1. Oxidative stress in *E. coli*

Understanding the oxidative stress response and cellular antioxidant defenses in *E. coli* is necessary before polyphenol antioxidant effects can be understood. Previous studies by Schellhorn *et al.* (*266*) and Imlay *et al.* (*19*) have shown that upon H_2O_2 challenge, oxidative DNA damage resulted in *E. coli* cell death. Imlay and coworkers found that this DNA damage was primarily from cellular Fe^{2+} generation of $^{\bullet}OH$, and labile Fe^{2+} concentrations were measured to be ~10 μM (*18*). Because iron-mediated DNA damage is also the primary cause of human cell death (*18*, *21*, *176*, *206*), *E. coli* studies of polyphenol antioxidant activity under oxidative stress conditions can serve as pioneer studies prior to human cell experiments. *E. coli* also has excellent anaerobic growth properties that allow scientists to create oxygen-sensitive mutants without oxidative stress defense enzymes to observe the impact of ROS on cellular functions (*262*, *267*).

As in human cells, ROS produced in *E. coli* cause oxidative stress, and *E. coli* responds to oxidative stress by invoking two distinct responses, the peroxide (H_2O_2) stimulon and the superoxide ($O_2^{\bullet-}$) stimulon (*179*). Oxygen molecules can move freely across *E. coli* cell membranes, so increased oxygen concentrations in air immediately raise intracellular O_2 concentrations (*262*, *268*). Intracellular O_2 can abstract an electron from electron-transfer enzymes such as flavoenzymes, thus generating $O_2^{\bullet-}$ and H_2O_2 (Figure 8) (*262*). Approximately 15 μM/s of H_2O_2 and 5μM/s of $O_2^{\bullet-}$ are produced in wild-type *E. coli* under ambient O_2 concentrations (*179*). Since ROS formation occurs naturally in *E. coli*, it is unsurprising that bacteria have strong defenses against ROS damage. *E. coli* oxidative stress defenses include antioxidant defense enzymes such as catalase and peroxidase (gene products of *katG* and *katE*) that decompose H_2O_2 (*269*). In addition to hydrogenperoxidase, *E. coli* cells grown under aerobic conditions generally have two superoxide dismutase (SOD) enzymes, Mn-containing SOD (MnSOD) and Fe-containing SOD (FeSOD), that dismutate $O_2^{\bullet-}$ to H_2O_2 and O_2 (*200*, *270–272*). Cellular H_2O_2 can react with non-protein-bound Fe^{2+} or oxidize iron-sulfur clusters in proteins ([4Fe-4S]) to generate hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction (Figure 8) (*179*). NADH peroxidase and catalase are responsible for most of the H_2O_2 scavenging in *E. coli*, scavenging H_2O_2 when concentrations are over 20 nM, to optimize H_2O_2 at a low steady state concentration (*200*, *270*, *271*). Upon H_2O_2 challenge, NADH peroxidase is saturated, and catalase becomes the primary scavenging enzyme (*179*, *262*). At normal H_2O_2 concentrations, the Fenton reaction is not the primary cause of endogenous oxidative stress in *E. coli*, but if H_2O_2 concentrations exceed 20 nM, $^{\bullet}OH$ generated from the Fe^{2+}-mediated Fenton reaction leads to oxidative DNA damage and cell death (*194*, *269*).

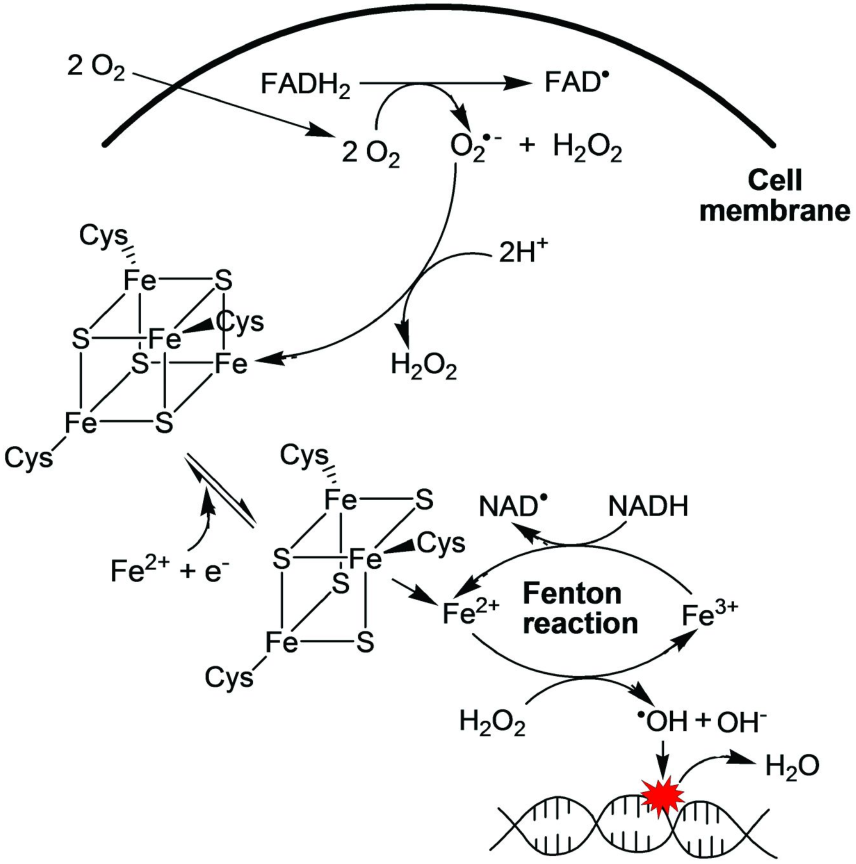


Figure 8. Possible mechanisms of ROS generation in E. coli.

Superoxide is another major ROS that is generated as a byproduct of the respiratory chain in *E. coli*. Superoxide cannot directly oxidize amino acids and nucleic acids but electrostatically binds to the catalytic iron atom of [4Fe-4S] clusters (*262*), especially the dehydrated $[4Fe\text{-}4S]^{2+}$ cluster of dihydroxyacid dehydratase (*19*, *194*, *262*). Upon $O_2^{\bullet-}$ binding, oxidation of the cluster occurs, generating an unstable $[4Fe\text{-}4S]^{3+}$ cluster that loses Fe^{2+} (Figure 8) (*194*, *273*). Free Fe^{2+} generates $^{\bullet}OH$ in the Fenton reaction and leads to oxidative DNA damage. Therefore, $O_2^{\bullet-}$ regulation is as important as H_2O_2 regulation, and

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

without oxidative stress defensive enzymes, ROS levels are unbalanced and lead to cell damage and death. For example, the *sodA sodB* (coding for MnSOD and FeSOD, respectively) double-mutant is an *E. coli* strain without SOD activity and has cellular $O_2^{\bullet -}$ concentrations of more than 2000 times those of wild-type cells (*272*). In addition, this *E. coli* strain was reported to be 30 times more sensitive to H_2O_2 challenge and was capable of fewer cell divisions compared to the parental strain (*274*, *275*).

4.2. Hydrogen Peroxide Regulation by the OxyR Regulon

The *E. coli* response to increased concentrations of H_2O_2 or organic peroxides such as *tert*-butyl hydrogen peroxide is defined as an aperoxide-mediated response, and stimulates nine proteins in the peroxide stimulon (*200*). These nine hydrogen-peroxide-inducible proteins are regulated by the OxyR regulon (Table 3). Unsurprisingly, without the OxyR regulon, *E. coli* strains are extremely sensitive to H_2O_2 (*270*). OxyR responds to oxidative stress and transduces the self-defense message to RNA polymerase (*179*, *270*) .

Table 3. Proteins regulated by the OxyR regulon (*270*)

Gene(s)	***Role***	***OxyR Response***
AhpC, AhpF *Catalase A* *Thioperoxidase*	H_2O_2 scavenging	AhpC, AhpF Catalase G
HemAXCDBL	Heme synthesis	Ferrochetalase
	FeS cluster assembly	SufABCDE
MrgA, Ferritin	Iron scavenging	Dps
Fur	Iron-import control	Fur

Although the mechanism of OxyR activation is still debated, most studies have shown that under oxidative stress, the OxyR regulon is oxidized and binds to recognition sequences of the OxyR protein at either Cys199 or Cys208 sites and oxidizes these thiol-containing residues to form a disulfide bond (Figure 9). The oxidized OxyR regulon then undergoes a conformational change, making it transcriptionally active. Activated OxyR transduces this oxidative stress signal to RNA polymerase to activate correlated genes such as *grxA* and *gorA* and also stimulates glutathione reductase that can reduce and inactivate OxyR (Figure 9) (*179*, *270*). Studies to identify new genes and pathways involved in cellular oxidative stress defense via the OxyR regulon are still underway. Since the OxyR regulon controls a major oxidative stress response, fully understanding its response mechanisms will also advance understanding of polyphenol antioxidant activity in *E. coli*.

4.3. Superoxide Regulation by the SoxRS Stimulon

The first enzyme identified to control $O_2^{\bullet -}$- mediated stress was MnSOD, and MnSOD was then found to be induced by the superoxide (SoxRS) stimulon that activates more than 20 genes (Table 4) (*179*, *273*). The superoxide stimulon is regulated by two major proteins, SoxR, a sensor protein which responds to oxidative stress, and SoxS, a transcriptional activator that triggers defensive mechanisms by activating response genes (*273*). When $O_2^{\bullet -}$-mediated stress occurs, SoxR is activated by oxidation of its [2Fe-2S] cluster, resulting in disorder in the SoxR protein structure. Activated SoxR then activates the SoxS promoter and increases SoxS expression (Figure 10) (*262*, *273*). This process increases SoxS synthesis, and SoxS activates target genes such as *sodA,* the gene that activates MnSOD expression to regulate oxidative stress, and *nfo*, the gene activates endonuclease IV (Endo IV) that triggers DNA repair process (Figure 10) (*193*).

E. coli oxidative stress defense mechanisms are not fully understood, but there has been rapid progress in this area due to the availability of *E. coli* mutants (*194*, *272*). Understanding oxidative stress defense mechanisms in *E. coli* will help dramatically in understanding oxidative stress defense pathways in human cells. Although oxidative responses and enzyme regulatory pathways in human cells are more complex than in *E. coli*, the basic cellular mechanisms can be similar and *E. coli* studies may be useful in some cases to identify biomarkers and cellular mechanisms for polyphenol antioxidant activity prior to human cell studies. Because cellular enzyme regulation mechanisms of polyphenol antioxidants can often overlap with ROS scavenging and metal chelation mechanisms, *E. coli* mutants can also be used to help distinguish between these three mechanisms.

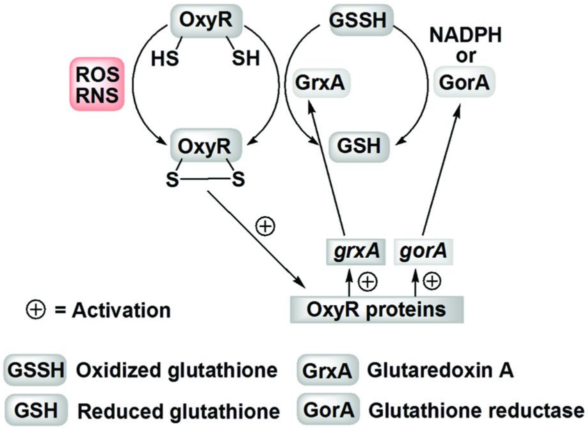


Figure 9. A proposed scheme for induction of the OxyR regulon in E. coli.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 4. Selected genes induced by the SoxRS stimulon (*179*, *273*)

Gene	*Enzyme activity*
Oxidant –resistant dehydratase isozymes	
fumC	Fumarase C
acnA	Aconitase A
Suspected [2Fe-2S] cluster repair	
yggX	Fe/S cluster repair protein
zwf	Glucose-6-phosphate dehydrogenase (G6PD)
fpr	NADPH:flavodoxin/ferredoxin oxidoreductase
fldA/B	Flavodoxin A/B
Drug efflux and/or resistance	
acrAB	Drug efflux pump
tolC	Omp component of drug efflux pump
micF	OmpF antisense sRNA
marAB	Multiple antibiotic resistance operon
nfnB	Nitroreductase
rimK	Modification of ribosomal protein S6

5. Mechanisms of Polyphenol Antioxidant Activity in *Escherichia coli*

Polyphenol antioxidant studies with *E. coli* can be traced back to 1951, when Mapson *et al.* attempted to determine how vegetable extracts affect the reduction of dehydroascorbic acid in this organism (*276*). The antioxidant behavior of polyphenols was not fully discussed in this study, but *E. coli* growth rates were altered upon adding plant extracts. Since then, more than 1300 papers describing polyphenol antioxidant research in *E. coli* have been published (*277*), demonstrating that polyphenols inhibit DNA and other cellular oxidative damage (*278–280*). In 2010, more than 800 papers were published on polyphenol research in *E. coli*, the majority of which are food-poisoning-related studies that examine polyphenol inhibition of *E. coli* O157:H7 growth (*281–283*). Polyphenol antioxidant research in *E. coli* has primarily focused on $O_2^{\bullet-}$ and H_2O_2 scavenging, overlooking other possible antioxidant mechanisms. Recent studies have shown that $NO^{\bullet}$ is toxic to *E. coli* due to its acceleration of $O_2^{\bullet-}$ formation and ability to oxidize polyphenol compounds to DNA-damaging quinone radicals (*267*). Additionally, peroxynitrite ($OONO^-$) is formed from the reaction of $NO^{\bullet}$ and $O_2^{\bullet-}$ and can cause oxidative stress in *E. coli* by direct oxidative damage as well as nitration of protein tyrosine residues that can alter enzyme function (*267*). The majority of polyphenol antioxidant studies in *E. coli* focus on ROS or RNS scavenging, with some attention to enzyme regulation. Recent studies have also

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

shown that polyphenol antioxidant activity is related to iron chelation. In this section, the evidence for and relationships between ROS and RNS scavenging, enzyme regulation, and metal chelation mechanisms for cellular polyphenol activity will be examined; polyphenol antibacterial effects will not be discussed.

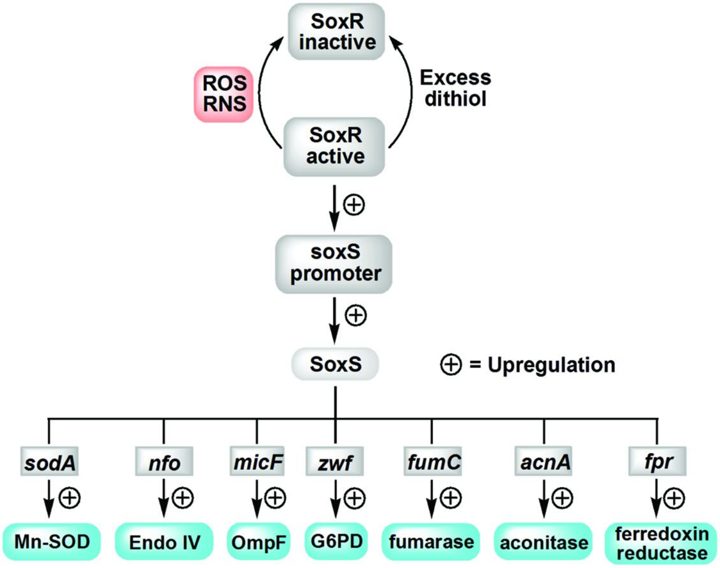


Figure 10. ROS and RNS regulation by the SoxRS stimulon.

5.1. Polyphenol Antioxidants Inhibit Oxidative Stress by ROS and RNS Scavenging

Several studies have shown that polyphenol compounds with catechol groups reduce ROS levels in *E. coli* with IC_{50} values of ~50 µM (*216*, *284*). Polyphenol compounds typically do not cause cell death in *E. coli* up to 200 µM, and, although cell growth rates slowed upon initial addition of polyphenols at high concentrations, normal growth rates resumed after 1 h (*216*). Galina *et al.* (*216*) tested the ability of quercetin, catechin, tannic acid, hesperetin, and naringenin (Figure 3) to prevent cell death from H_2O_2 challenge (2 mM) in 12 *E. coli* mutant strains unable to induce genes responding to peroxide stress. Quercetin, catechin, and tannic acid were the most effective ROS scavengers, with IC_{50} values for cell death prevention from 5 – 19 µM, values significantly lower than found for Trolox (IC_{50} = 40 µM). Naringenin and hesperetin were much less effective ROS scavengers, with IC_{50} values from 200-1000 µM, values almost 100 times less than those for the polyphenol compounds containing catechol groups (*216*). This study is one of the few that considered ROS scavenging, enzyme regulation, and iron-chelation mechanisms simultaneously. Although the authors stated that a full understanding of the cellular mechanisms for polyphenol antioxidant activity remained uncertain and required further investigation, this study marks a turning point in polyphenol antioxidant research.

Park *et al.* tested twenty-seven polyphenol compounds for ROS scavenging activity in *E. coli*, including quercetin, rutin, and kaempferol (*285*). Polyphenol antioxidants (10 μM) were added to cell cultures over 35 days, and cell survival rates were determined by colony counting. Although no oxidative stress was applied, rutin, quercetin, and kaempferol increased cell survival 100% to 200% compared to non-treated cells (*286*). To identify the mechanisms responsible for this increased cell survival, Park and coworkers used mutant strains to examine cellular enzyme regulation and *in vitro* iron-mediated oxidative DNA damage inhibition studies to measure ROS scavenging. Although the authors concluded that the polyphenols promoted *E. coli* survival primarily by ROS scavenging (*285*), since this conclusion was based on comparing cellular results with *in vitro* iron-mediated DNA damage assays, it is not clear whether polyphenol antioxidants inhibited DNA damage through ROS scavenging or iron chelation. In addition, correlations of the *in vitro* DNA damage results with cell survival results were not high, perhaps due to differing bioavailabilities of the tested polyphenols. Such uncertainties in cellular mechanistic studies are representative of the issues encountered when interpreting the polyphenol antioxidant literature.

Oktyabrsky *et al.* (*268*) tested several polyphenol-containing plant extracts (including extracts from *Rosa majalis*, *Sanguisorba officinalis*, and *Fragana vesca*) for their ability to prevent DNA damage and cell death in *E. coli* upon H_2O_2 challenge. Cell viability was assessed by colony counting, a DNA strand breakage assay was used to determine total antioxidant capacity, and the DPPH scavenging assay was used to determine ROS scavenging ability (*268*). In this study, polyphenol promotion of cell viability correlated very well with results from the DPPH scavenging assay ($R^2 = 0.95$) but not as well with results of an iron binding assay ($R^2 = 0.69$), indicating that these plant extracts inhibited cell death primarily by ROS scavenging rather than iron binding (*268*).

Antioxidant activities of *Moricandia arvensis* extracts in wild-type and DNA repair mutant (*uvrA*) *E. coli* strains were determined by Skandrani *et al.* (*287*) Cell cultures were challenged with nitrofurantoin (NF, 400 μM) or H_2O_2 (5 mM) and genotoxicity assays were performed to evaluate antioxidant ability. Extract treatment (1 μg/μL) inhibited genotoxicity by 61% in wild-type and 78% in *uvrA* mutant cells upon NF challenge and 88% and 64%, respectively, upon H_2O_2 challenge. Genotoxicity prevention was very strongly correlated to the polyphenol content of the extracts ($R^2 = 0.99$), and polyphenol antioxidant activity was ascribed to ROS scavenging by comparisons with results from *in vitro* DPPH and Trolox scavenging assays. The authors stated that the polyphenol-containing extracts inhibited H_2O_2-induced oxidative stress because polyphenol compounds are effective hydrogen donors that inhibit ROS production by H_2O_2 oxidation (*287*). However, since the polyphenol extracts showed 27% greater prevention of genotoxicity upon H_2O_2 challenge compared to NF challenge in the wild-type strain, and H_2O_2 generates DNA-damaging $^{\bullet}OH$ *via* the iron-mediated Fenton reaction, iron chelation may also have significantly contributed to the observed antioxidant activity. In this study, as is common in polyphenol antioxidant

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

research, ROS scavenging is reported to be the major antioxidant mechanism without examining alternate possibilities.

Several reports state that no direct experimental evidence has proven the existence of polyphenol antioxidant mechanisms besides ROS scavenging, possibly leading researchers to neglect testing for additional mechanisms (*200*, *261*, *268*, *271*, *288*). Additionally, ROS scavenging assays are generally designed for *in vitro* studies and are often not suitable for cellular experiments (*287*), preventing direct *in vitro* and cellular comparisons. The source of radical generation should also be carefully considered when comparing results from cellular and *in vitro* experiments: *E. coli* contains ~10 μM of endogenous non-protein-bound Fe^{2+} (*179*, *194*), so H_2O_2 challenge generates ROS mainly through an iron-mediated pathway (*194*). Thus, when performing cellular H_2O_2 challenge experiments, iron chelation must also be examined as a possible polyphenol antioxidant mechanism. If researchers do not test and compare polyphenol antioxidant mechanisms other than ROS scavenging, the antioxidant potency of polyphenols can be under- or overestimated. Instead, several possible mechanisms should be tested and compared to draw accurate conclusions about polyphenol antioxidant activity in *E. coli*.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

5.2. Polyphenol Antioxidants Inhibit Oxidative Stress by Enzyme Regulation

In addition to ROS scavenging, enzyme regulation has also been examined as a potential mechanism for polyphenol antioxidant activity using *E. coli* mutant strains. In *E. coli*, most studies have focused on polyphenol regulation of OxyR and MnSOD because these represent the two major enzymatic pathways for enzymatic defense, the OxyR regulon and the SoxR stimulon, and because appropriate *E. coli* mutants are available for comparison studies.

Smirnova *et al.* (*216*) tested the antioxidant activity of quercetin, tannin, catechin, naringenin, and hesperetin (Figure 3) in *E. coli* antioxidant defense mutant strains to evaluate polyphenol enzyme regulation. Mutant catalase-hydroperoxydase I expression (*katG*), alkylhydroperoxide reductase (*ahp*) and iron uptake regulator (*fur*) *E. coli* strains were tested to determine polyphenol effects on OxyR regulation. Mutant SoxRS stimulon (*soxS*) and MnSOD (*sodA*) strains were also tested for cell growth and survival effects upon polyphenol treatment (*216*). Upon H_2O_2 challenge (10 mM for 30 min), wild-type *E. coli* pre-treated with quercetin and tannin (100 μM) showed significant cell death inhibition compared to untreated cells. When these cellular effects were compared to results from *in vitro* studies of ROS scavenging by Trolox and iron chelation by bipyridine and deferoxamine, only deferoxamine treatment showed similar ROS scavenging and iron chelating effects (*216*). Compared to the wild-type strain, the *oxyR* mutant strain pre-treated with quercetin or deferoxamine and then challenged with H_2O_2 showed 100-fold less cell death prevention, indicating that OxyR-regulated enzyme activities are essential for the antioxidant effects of quercetin (and deferoxamine). Under similar conditions, quercetin prevented five-fold less cell death in the *katG* mutant strain compared to the wild-type strain, suggesting that *katG* expression was also involved in the protective effects of quercetin (*216*). Overall, the authors concluded that quercetin and tannin were the only polyphenol compounds to promote cell survival upon H_2O_2 challenge, and increased cell survival was due to polyphenol up-regulation of catalase activity. Polyphenol antioxidants prevented cell death less effectively in the *oxyR* mutant, suggesting that polyphenol compounds activated OxyR transcriptional regulation to increase antioxidant defense gene expression and inhibit oxidative stress (*216*).

In the same study (*216*), the superoxide generators menadione and paraquat were used to induce the *E. coli* SoxRS response, and the *sodA* mutant pretreated with quercetin, tannin, bipyridine, or deferoxamine increased β-galactosidase expression by two-fold compared to non-treated cells. Since β-galactosidase is produced by SoxRS activation, increased β-galactosidase levels indicated that these polyphenol compounds increased gene expression upon ROS challenge. Polyphenol antioxidants also induced *sodA* expression upon superoxide challenge, and this increase in expression likely resulted from a decrease in iron concentration due to polyphenol-iron binding, since treatment with the iron chelators bipyridine and deferoxamine showed similar results (*216*). Another strong metal chelating polyphenol, catechin, showed no protective effects upon superoxide challenge. Because catechin crosses cell membranes at a lower rate than quercetin, these differences in antioxidant activity may result from its lack of cellular bioavailability (*55*, *56*). Cellular Trolox assays showed no protective effects on cell survival, either due to its low bioavailability (*55*, *289*), or the fact that ROS scavenging was not a major antioxidant mechanism compared to enzyme regulation and metal chelation. This study is one of the few to test and compare all three major polyphenol mechanisms, and the results highlight the close relationship between enzyme regulation and metal chelation antioxidant mechanisms.

In a pioneer study, Park *et al.* (*286*) examined twenty-seven polyphenol compounds, including catechin, morin, rutin, kaempferol, and quercetin, for the ability to prevent cell death in *E. coli soxS(p)-lacZ*, *oxyS(p)-lacZ*, and *sodA* mutant strains under oxidative stress. Upon paraquat (100 μM) and H_2O_2 (50 μM) challenge, the *soxS(p)-lacZ* mutant only survived after superoxide challenge, whereas the *oxyS(p)-lacZ* mutant only survived after H_2O_2 challenge. Supplementation of *oxyS(p)-lacZ* mutant cell cultures with quercetin, rutin, and kaempferol (50-1000 μM) increased cell survival upon H_2O_2 challenge in a dose-dependent manner. Under similar conditions, catechin supplementation promoted cell survival by only 15%, and other polyphenol compounds showed no effect (*286*). Polyphenol supplementation (500 μM) also showed little ability to increase *soxS(p)-lacZ* mutant cell survival upon superoxide challenge (*286*, *290*). Because oxidative challenge was initiated immediately after polyphenol addition, and previous studies reported that polyphenol compounds require up to 2 h to cross the cell membrane and achieve maximal cellular concentrations (*193*, *290*, *291*), it is likely that intracellular polyphenol concentrations in this study were low.

An additional cell survival experiment with the *sodA* mutant showed that quercetin, rutin, and kaempferol supplementation (10 μM) without oxidative challenge effectively increased cell survival over 35 days by more than 50% compared to non-treated cells, indicating that polyphenols may have increased

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

cell survival via *sodA* enzyme expression regulation through the SoxRS stimulon (*285*). In this study, ROS scavenging and enzyme regulation were considered as major polyphenol antioxidant mechanisms. Metal chelation was also mentioned, but the authors stated there was no direct evidence to prove this mechanism. The authors reported a method to quickly screen polyphenol compounds for antioxidant ability, but additional studies were needed to fully elucidate polyphenol cellular mechanisms.

From *E. coli* studies, researchers have identified several important enzymes involved in antioxidant defenses, including SOD, catalase, and DNA repair enzymes (*179*, *194*, *262*). Polyphenol antioxidant studies of enzyme regulation in *E. coli* have shown that polyphenol antioxidants up-regulate the OxyR regulon and reduce ROS generation (*266*, *268*, *285*, *292*). However, polyphenol antioxidants also have strong metal binding abilities that can reduce endogenous non-protein-bound iron concentrations and therefore ROS generation. Since oxidative-stress-defense enzymes are triggered by high ROS concentrations (*179*), polyphenol enzyme regulation and iron chelation mechanisms are interrelated, making independent studies of these cellular mechanisms difficult.

5.3. Polyphenol Antioxidants Inhibit Oxidative Stress by Metal Binding

The iron chelating ability of polyphenol antioxidants has attracted much recent interest as a possible antioxidant mechanism. Imlay *et al.* (*18*, *19*) demonstrated that *E. coli* exposed to H_2O_2 (2.5 mM) caused DNA damage and cell death due to iron-mediated $^{\bullet}OH$ generation (Reaction 1), and this iron-mediated damage was the main cause of *E. coli* cell death under these conditions. Recently, several studies have strongly supported the concept that polyphenol-iron binding inhibits $^{\bullet}OH$ generation, resulting in increased cell survival upon polyphenol supplementation (*156*, *245*, *268*, *293*).

Polyphenols bind transition metals (including Fe^{2+}) and inhibit $^{\bullet}OH$ generation, reducing oxidative stress (*29*, *156*, *216*, *245*). When Fe^{2+} binds polyphenols, oxidation to the Fe^{3+}-polyphenol complex occurs, resulting in the appearance of a charge-transfer band (~450 nm) in the visible spectrum (*156*, *245*). Smirnova *et al.* (*216*) used spectroscopic methods to measure the iron chelating properties of a variety of polyphenols, and compared these results with that of ferrozine, a known iron chelator. Polyphenol compounds with iron-binding catechol groups such as quercetin, tannic acid, and catechin demonstrated better *in vitro* iron chelating abilities compared to polyphenol compounds without catechol groups. Quercetin, tannin, and catechin also chelated iron similarly to or better than the well-studied iron chelators bipyridine and deferoxamine (*216*).

In addition to *in vitro* assays to measure polyphenol-iron binding abilities, Smirnova and coworkers (*216*) measured the effects of polyphenol supplementation (200 μM for 30 min) on *fur* regulated *iuc::lacZ* expression in *E. coli* upon H_2O_2 challenge (10 mM for 30 min) to determine intracellular polyphenol-iron chelation. The *fur* mutant strain cannot regulate iron uptake, resulting in an eight-fold increase in labile iron compared to wild-type cells (*294*). Quercetin and tannic acid were the only two tested polyphenols capable of strong iron binding, and they promoted cell survival upon H_2O_2 challenge

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

by ~50% compared to untreated cells. Because these polyphenols showed similar increases in cell survival compared to cells treated with the iron chelators bipyridine (60%) and deferoxamine (49%), the authors concluded that quercetin and tannic acid exerted their protective effects by binding intracellular iron (*216*). Interestingly, both deferoxamine and tannic acid bound iron five times more strongly than quercetin *in vitro,* but showed weaker intracellular iron chelating ability as measured by iron uptake chelate (*iuc*) gene expression levels that are altered by reduced labile iron levels arising from iron chelation. These differences between *in vitro* and cellular results may have reflected differences in membrane permeability of the tested polyphenols (i.e., differences in ionization as measured by polyphenol pK_a values) that affect cellular bioavailability. Overall, a strong correlation between intracellular iron chelation ability and antioxidant potency of polyphenol compounds led the authors to conclude that intracellular polyphenol-iron binding was a primary mechanism against H_2O_2 induced oxidative stress in *E. coli* (*216*).

In similar experiments (*216*), the effects of polyphenol supplementation (200 µM for 30 min) on the *sodA* mutant *E. coli* strain upon superoxide challenge (200 µM menadione for 30 min) showed that quercetin, tannic acid, bipyridine, and deferoxamine increased β-galactosidase expression by intracellular iron binding. These results agreed with previous reports that iron chelators increased *sodA* expression by binding labile iron within the cell, causing iron deficiency and inactivating the ferric uptake regulator (Fur) protein (*295*). The authors concluded that ROS scavenging, enzyme regulation, and iron chelation were three possible mechanisms for polyphenol antioxidant activity in *E. coli*, but that iron binding and enzyme regulation had significant overlap and were therefore difficult to assess individually (*216*).

Park *et al.* (*285*) tested the cellular iron binding properties of 27 polyphenols in *E. coli* by adding the reductant dithiothreitol (DTT, 10 mM) and then $FeCl_3$ (400 µM) into polyphenol-supplemented cell cultures. In the presence of DTT, Fe^{2+} was generated in the culture medium and increased iron-mediated cell death. After addition of the polyphenol compounds (10 µM) to cultures of a superoxide dismutase (*soxS-lacZ*) mutant, the cultures were allowed to stand at room temperature for 35 days as cell survival was monitored. Among the tested polyphenols, rutin, quercetin, and kaempferol promoted *E. coli* cell survival up to 50% compared to non-treated cells, indicating that these polyphenol compounds may chelate iron to restore cell viability (*286*). *In vitro* gel electrophoresis experiments showed that only quercetin inhibited iron-mediated DNA damage. Although the authors mentioned that metal binding may be an essential polyphenol antioxidant mechanism, no direct experimental evidence for metal binding was presented in this study (*285*). Since the polyphenol antioxidants were added simultaneously with DDT and the $FeCl_3$ was added only after 30 min, it is not possible to determine whether quercetin inhibited DNA damage through ROS scavenging, iron chelation, or both.

Oktyabrsky and coworkers (*268*) used spectrophotometry to determine the iron chelating abilities of different plant extracts (including *Rosa majalis, Sanguisorba officinalis*, and *Fragana vesca*), but found that ROS scavenging ability (as measured by the DPPH assay) of the extracts showed a stronger correlation with prevention of H_2O_2-induced cytotoxicity in *E. coli* than iron binding ability. The authors therefore concluded that iron chelation may be a major polyphenol antioxidant mechanism, but cell death prevention in *E. coli* occurred primarily through ROS scavenging (*268*). Given these conflicting reports of ROS scavenging, enzyme regulation, and iron binding as primary mechanisms of polyphenol antioxidant activity in *E. coli*, and the lack of cellular bioavailability data to properly correlate *in vitro* and cellular polyphenol antioxidant activity, it is clear that additional investigations are required to determine the relative contributions these three cellular antioxidant mechanisms.

Generally, polyphenol compounds with a catechol group on the B ring showed greater ROS scavenging and metal chelation antioxidant abilities than polyphenols without catechol groups (*244*, *247*, *258*, *296*), but comparisons between gallol and catechol-containing polyphenols are difficult due to their differing solubilities (*154*, *155*). Recently, researchers have begun to test and compare multiple mechanisms for polyphenol antioxidant activity, instead of focusing on only one, although some authors claim that iron binding should not be considered a major antioxidant mechanism due to the limited solubility of polyphenol compounds with the strongest iron binding abilities (*154*). Understanding how polyphenol ionization affects bioavailability in cellular experiments is essential, since correlations between *in vitro* and cellular experiments require accurate determinations of intracellular polyphenol concentrations. In addition, the ease of working with *E. coli*, the ready availability of *E. coli* mutant strains, and the similarity of *E. coli* and human cellular responses to oxidative stress will help to identify possible pathways affected by polyphenols, significantly advancing mechanistic studies of polyphenol antioxidant behavior in human cells.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

5.4. Polyphenol Compounds as Prooxidants

Many *in vitro* studies have shown that polyphenol compounds also inhibit copper-mediated DNA damage, but that they interact with copper differently compared to iron (*244*, *247*, *297*, *298*). Because of these differences, it is unclear whether polyphenol antioxidants inhibit cellular DNA damage by copper chelation. In addition to antioxidant activity, polyphenols can act as prooxidants in *E. coli*, especially in the presence of $NO^\bullet$ and copper (*292*, *299*, *300*). Polyphenols can scavenge $NO^\bullet$, but this $NO^\bullet$ scavenging generates quinone radicals that can cause cellular damage (*29*, *301*). Urios *et al.* (*292*) demonstrated that polyphenol compounds (1-15 mM) increase *E. coli* cytotoxicity by 50% in the presence of $NO^\bullet$ compared to $NO^\bullet$-treated cells without polyphenol supplementation. *OxyR*$^-$ and *OxyR*$^+$ *E. coli* mutants were used to determine the mechanisms for this polyphenol cytotoxicity by treating these mutant strains with catechol and hydroquinone compounds (50-2000 μg/plate) and the same concentrations of diethylamine/NO (DEA/NO), an $NO^\bullet$ donor. Previous studies had shown that under these conditions, phenolic compounds such as catechols and catecholamines acted as oxidative mutagens in *OxyR*-deficient strains, undergoing autoxidation to quinone radical species and producing ROS that caused cellular toxicity *via* multiple pathways (*302*). The tested catechol and hydroquinone compounds also showed DNA damaging and cytotoxic effects in the *OxyR+* strain that could not be eliminated by antioxidant enzymes such as catalase and alkyl hydroperoxide reductase, indicating that the toxic effects were possibly mediated by quinone radical (*292*). In addition, quercetin, EGCG, kaempferol, and luteolin (50-2000 µM) were found to be prooxidants in the presence of Cu^{2+}, causing site-specific DNA damage *in vitro* (*303*). To cause this DNA damage, the polyphenol was believed to bind Cu^{2+} and oxidize, releasing quinone radical and forming Cu^{+}-DNA adducts capable of $^{\bullet}$OH generation (Figure 11). Polyphenols also have the ability to regenerate intracellular Fe^{2+} by inducing riboflavin reductase gene (*fre*) expression, resulting in increased intracellular Fe^{2+} concentrations and promoting the formation of iron-generated $^{\bullet}$OH (*304*).

Figure 12 summarizes known polyphenol antioxidant and prooxidant mechanisms in *E. coli*. The cytotoxic properties of polyphenols are still under debate, since the majority of studies have demonstrated no cytotoxic properties up to 200 µM (*193*, *200*, *268*, *280*), and studies reporting polyphenol prooxidant activity are often at very high concentrations (~1-15 mM) or are accompanied by RNS treatment (*292*, *302*, *305*). Examining and correlating the relative contributions of polyphenol antioxidant (and prooxidant) mechanisms in cells is a complex undertaking, especially given potential correlations and interrelationships between ROS scavenging, enzyme regulation, and metal chelation mechanisms. To date, polyphenol antioxidant research has mainly focused on ROS scavenging and enzyme regulation, but studies have also shown that iron chelation can have a major impact on polyphenol cellular effects. Comparing results from *in vitro* assays and *E. coli* cell studies will aid in identifying possible polyphenol antioxidant mechanisms and provide critical data for comparing polyphenol effects in the more complex environment of human cells.

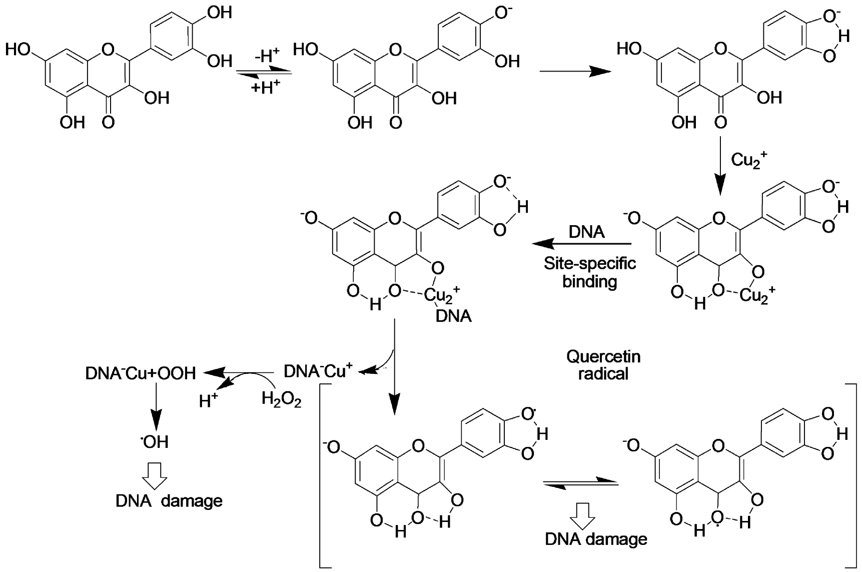


Figure 11. A proposed mechanism for quercetin-induced cytotoxicity in the presence of Cu^{2+} (303).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

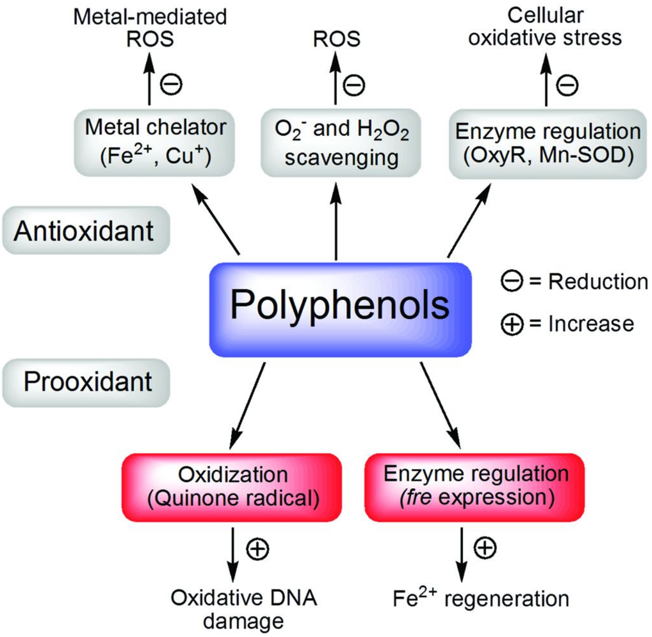

Metal-mediated ROS
ROS
Cellular oxidative stress
⊖
⊖
⊖
Metal chelator (Fe^{2+}, Cu^{+})
O_2^- and H_2O_2 scavenging
Enzyme regulation (OxyR, Mn-SOD)
Antioxidant
Polyphenols
⊖ = Reduction
⊕ = Increase
Prooxidant
Oxidization (Quinone radical)
Enzyme regulation (*fre* expression)
⊕
⊕
Oxidative DNA damage
Fe^{2+} regeneration

Figure 12. Summary of polyphenol antioxidant and prooxidant mechanisms in E. coli.

6. Oxidative Stress and Antioxidant Defenses in Human Cells

In human cells, excess levels of ROS or RNS induce oxidative stress, and the resulting oxidative damage can cause permanent DNA modifications, resulting in mutagenesis, carcinogenesis, and cell death (*8*, *185*, *306*). It has been reported that a single human cell is exposed to about 1.5 × 10^5 hydroxyl radicals or other reactive species a day (*188*). Hydroxyl radical can damage both purine and pyrimidine bases as well as the deoxyribose backbone of DNA, and more than 100 oxidative DNA damage products have been identified, including 8-oxo-dG and 8-nitroguanine (*190*, *300*). DNA damage can alter cellular transcription, signal transduction, and DNA replication, causing genomic instability and leading to disease development (*148*, *188*). In addition to the intensively studied nuclear DNA damage, mitochondrial DNA damage can also occur in human cells (*138*, *176*). In fact, mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA because mitochondria produce the majority of ROS, and mitochondrial DNA is not protected by histone proteins (*307*, *308*). DNA repair mechanisms can correct nuclear DNA damage (*10*, *176*, *206*); mitochondrial DNA repair also occurs, but these repair mechanisms remain unclear (*138*, *309*). Since the capacity for mitochondrial DNA repair is lower than for nuclear DNA repair, mitochondrial oxidative stress reduction is reliant primarily upon preventative mechanisms such as ROS scavenging and enzymatic detoxification (*11*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

In addition to DNA damage, oxidative stress also results in lipid peroxidation and protein oxidation (*9*, *10*). Iron-generated hydroxyl radical (Reaction 1) is proposed to be the primary cause of this oxidative damage (*66*). Radical generation is tightly linked to the participation of redox-active metals, and cellular redox balance requires maintaining metal ions and ROS/RNS within strict physiological limits (*11*, *18*). It has been reported that this iron regulation ensures that there is no labile intracellular iron in human cells (*271*, *273*). In contrast, Gao *et al.* and other groups have measured intracellular non-protein-bound iron concentrations of ~1-10 μM in various human cells lines, similar to labile iron levels in *E. coli* (*18*, *176*, *310*, *311*). Under oxidative stress conditions in human cells, as in *E. coli*, excess superoxide can oxidize [4Fe-4S] cluster-containing enzymes, releasing additional labile iron that can generate highly reactive $^{\bullet}OH$ (*11*, *254*). Several *in vivo* studies have shown that after oral intake of iron carbonyl, oxidation of polyunsaturated fatty acids, lipids, and proteins occurred in rats (*204*, *256*, *312*, *313*). These observations have suggested a direct relationship between iron-mediated oxidative damage and development of inflammation, arteriosclerosis, cancer, and Parkinson's disease (*312*, *314*, *315*).

To control ROS and RNS levels and to protect against oxidative damage, human cells contain several ROS/RNS scavenging and detoxifying enzymes. Although ROS/RNS are generated by different cell types *via* different enzymatic and non-enzymatic pathways, the basic mechanisms are similar. ROS and RNS form intermolecular disulfide bonds between proteins by oxidizing cysteine residues (*316*), and the structural changes resulting from these disulfide cross-links lead to increased levels of several cell signaling proteins, including growth factor kinase, mitogen-activated protein kinase (MAPK), proto-oncogenic tyrosine kinase (src), and phosphoinositide 3-kinase (PI3K). This cell signaling up-regulation activates several redox-regulated transcription factors such as activator protein 1 (AP-1), NF-κB, tumor protein 53 (p53), hypoxia-inducible factors (HIFs) and nuclear factor of activated T-cells (NFAT) (Figure 13, Table 5). Cellular enzymatic defenses directly remove ROS and RNS to protect against oxidative stress, but in addition to endogenous defenses, exogenous polyphenol antioxidants can also reduce ROS/RNS levels by ROS scavenging, enzyme regulation, and metal chelation mechanisms to prevent cellular damage.

7. Mechanisms for Polyphenol Antioxidant Activity in Human Cells

High ROS and RNS levels in human cells result in DNA damage, lipid peroxidation, and cell signaling up-regulation (Figure 5). Antioxidant defenses to this oxidative stress occur through both enzymatic and non-enzymatic pathways. Enzymatic pathways mainly involve superoxide dismutase, catalase, and glutathione peroxidase, enzymes that can directly decompose ROS (*5*, *354*). Non-enzymatic antioxidants are typically small molecules that inhibit hydroxyl radical generation or act as sacrificial oxidants to neutralize damaging reactive species (*107*, *126*, *169*, *259*, *268*). Interest in polyphenol antioxidants has recently increased as a result of increasing evidence of their beneficial impacts on human health and because they participate in both enzymatic and non-enzymatic defense pathways (*3*, *94*, *122*, *237*). Polyphenol antioxidants can chelate cellular redox-active metal ions such as $Fe^{2+/3+}$, $Cu^{+/2+}$, $Cr^{3+/4+}$, $CO_{2+/3+}$ and $V^{3+/4+}$ (*156*, *214*, *239*, *355*). In addition, polyphenol compounds interfere with oxidation of lipids and other biomolecules by reacting with damaging radical species to form a relatively stable phenoxy radical to inhibit further damage (Reaction 2) (*356*). Polyphenol compounds also modulate cell signaling, and studies have shown that polyphenols inhibit NF-κB, Akt, p53, and endothelial nitric oxide synthase (eNOS) regulation in rats (*168*, *357*). As previously discussed, the key structural properties that affect polyphenol antioxidant abilities include the number of hydroxyl groups, position of substituents, and glycosylation (*25*, *164*). All of these factors affect whether polyphenol compounds will act as ROS scavengers, metal chelators, or modulators of cell signaling (*146*, *244*, *259*). Under normal conditions, polyphenol compounds cause little cytotoxicity up to 400 μM (*52*, *358*), and several studies in a variety of human cell lines have shown significant polyphenol antioxidant effects via ROS scavenging, enzyme regulation and metal chelation mechanisms (*43*, *52*, *115*, *349*, *359*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

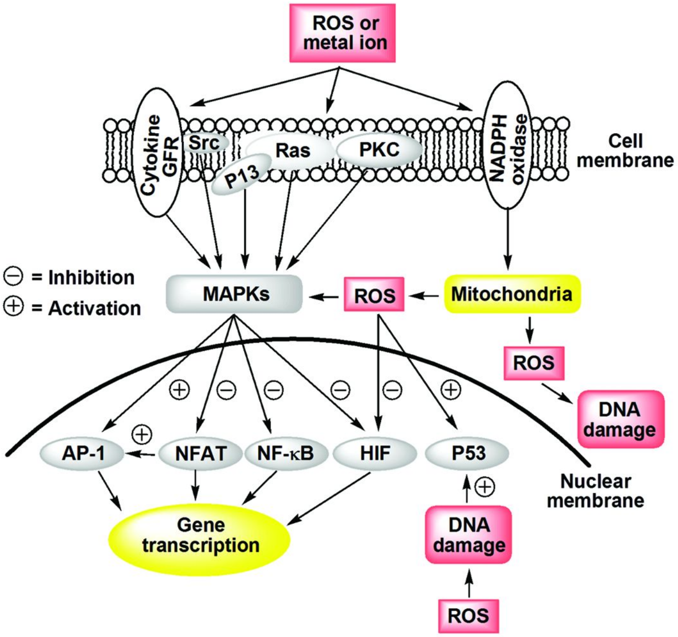

ROS or metal ion
Cytokine GFR
Src
P13
Ras
PKC
NADPH oxidase
Cell membrane
⊖ = Inhibition
⊕ = Activation
MAPKs
ROS
Mitochondria
ROS
DNA damage
⊕
⊖
⊖
⊖
⊖
⊕
⊕
AP-1
NFAT
NF-κB
HIF
P53
⊕
Nuclear membrane
Gene transcription
DNA damage
ROS

Figure 13. ROS/RNS- and metal-ion-induced signaling pathways in human cells.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 5. Effects of polyphenol antioxidants on inflammatory markers and signal transaction pathways

Target	***Polyphenol Compounds***	***References***
Inflammatory markers		
Endothelial nitric oxide synthase (eNOS)	quercetin, catechin, cyanidin-3-glucoside	(*240*, *317*)
Inducible NOS (iNOS)	quercetin, apigenin, rutin, wogonin, quercetin gallate, kaempferol, luteolin	(*318–323*)
Cyclooxygenase -2 (COX-2)	apigenin, rutin, wogonin, quercetin, kaempferol, luteolin, genistein, green tea polyphenols, curcumin,	(*168*, *318–326*)
5-Lypooxygenase (5-LOX)	apigenin, luteolin, curcumin	(*327–329*)
Interleukin (IL-family)	luteolin, apigenin, green tea polyphenols, quercetin, kaempferol, butein	(*247*, *323*, *330–332*)
Tumor necrosis factor (TNF-α)	quercetin, kaempferol, EGCG, luteolin, curcumin	(*323*, *333–337*)
Monocyte chemoattractant protein (MCP-1)	apigenin, EGCG	(*338*, *339*)

Continued on next page.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 5. (Continued). Effects of polyphenol antioxidants on inflammatory markers and signal transaction pathways

Target	***Polyphenol Compounds***	***References***
Inflammatory markers		
Intercellular adhesion molecule (ICAM-1)	flavonols, hesperidin, phellopterin, quercetin, kaempferol	(*338*, *340*)
Vascular cell adhesion molecule (VCAM-1)	flavonols, hesperidin, phellopterin, quercetin, kaempferol	(*338*, *340*)
Cell signaling pathways		
Vascular cell adhesion molecule (NF-κB)	quercetin, liquiritigenin, kaempferol, procyanidins, curcumin, apigenin	(*13*, *319*, *341*)
Activator protein (AP-1)	luteolin, kaempferol, quercetin, catechins, curcumin	(*73*, *115*, *323*, *330*, *342–344*)
Signal transducer and activator of transcription (STAT-1)	genistein, kaempferol, quercetin, daidzein, theaflavin, apigenin, luteolin	(*52*, *246*, *317*, *333*, *345*)
Peroxisome proliferator-activated receptor (PPAR-α)	EGCG, flavone	(*162*, *346*, *347*)
Hypoxia-inducible factor (HIF-1)	hesperidin, baicalein, quercetin, catechin, curcumin	(*215*, *219*, *260*, *318*, *319*, *323*)
Mitogen-activated protein kinase (MAPKs)	quercetin, luteolin, wogonin, apigenin, luteolin, kaempferol, chrysin, EGCG, hesperidin, curcumin	(*54*, *76*, *113*, *239*, *348–353*)

Polyphenol antioxidants are reported to prevent or ameliorate oxidative stress and disease, including liver diseases, obesity, cancer, cardiovascular diseases, Parkinson's and Alzheimer's diseases, diabetes, and osteoporosis (*130*, *166*, *189*, *360*). Polyphenols also have been reported to regulate over 20 enzymes through complex cellular mechanisms that control disease development. This section will focus on human cell studies of polyphenol antioxidants related to cancer, cardiovascular diseases, and neurodegenerative diseases (*6*, *24*, *130*, *203*, *361*). Cellular ROS scavenging, enzyme regulation, and metal chelation mechanisms for polyphenol antioxidant activity, as well as the interrelationships among these mechanisms, will also be discussed in detail. The prooxidant properties of polyphenols will be briefly discussed at the end of this section.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

7.1. Polyphenol ROS and RNS Scavenging Mechanisms in Human Cells

Polyphenol antioxidants are effective ROS and RNS scavengers due to their hydrogen donating abilities (*363*, *364*). Polyphenol antioxidants have favorable reduction potentials (0.75-0.33 V) compared to polyunsaturated fatty acids (1.0 V) and alkyl peroxy radicals (2.3-1.0 V) that allow them to effectively inhibit lipid peroxidation (Table 6) (*365*). Galleano *et al.* (*365*) have thoroughly reviewed polyphenol reduction potentials compared to other antioxidants and reducing agents and discussed the implications of polyphenol redox properties on inhibition of lipid peroxidation. Polyphenol prevention of lipid peroxidation and the general structural trends for this activity are also discussed in several papers (*211*, *212*, *362*).

Polyphenols with gallol groups have lower reduction potentials than their catechol analogs (*212*, *362*, *366*), and polyphenol reduction potentials correlated with results from several cellular studies that showed myricetin and quercetin inhibited lipid peroxidation more effectively than other polyphenols (*366*). Among tea polyphenols, EGCG and EGC inhibited lipid peroxidation significantly more than other catechin polyphenols (*107*, *168*). Sorata *et al.* (*367*) showed that quercetin and rutin (100-1000 μM) significantly reduced photosensitized hemolysis and lipid peroxidation of human erythrocytes caused by hematoporphyrin and lauroyl peroxide and were themselves oxidized during this process. Quercetin and rutin also showed dose-dependent *in vitro* $O_2^{\bullet-}$ scavenging ability. Correlation between cellular and *in vitro* results indicated that these polyphenols functioned as sacrificial ROS scavengers to terminate radical chain reactions and inhibit lipid peroxidation. In addition, quercetin and rutin antioxidant ability strongly correlated with their cellular bioavailability (*367*). Other studies using EPR measurements confirmed that polyphenols scavenge $O_2^{\bullet-}$, but rate constants for this scavenging reaction were difficult to determine (*215*, *368*). Taken together, these results indicate that polyphenol antioxidants inhibit cellular lipid peroxidation primarily by scavenging lipid radical species.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Table 6. Reduction potentials ($E^{1/2}$ vs. NHE) for lipid hydroperoxide, radical scavengers, and polyphenol compounds (*362*)

Compounds (Redox Couple)	*$E^{1/2}$ (mV)*
Lipid hydroperoxide (LOO$^{\bullet}$/LOOH)	1000
Glutathione-cysteine (RS$^{\bullet}$/RS^{-})	920
Trolox (RO$^{\bullet}$/ROH)	480
Ascorbate (Asc$^{\bullet}$/Asc)	282
Quercetin (Q$^{\bullet}$/Q)	330
(-)Epigallocatechin (EGC$^{\bullet}$/EGC)	430
(-)Epigallocatechin gallate (EGCG$^{\bullet}$/EGCG)	430
Catechol (Cat-O$^{\bullet}$/Cat-OH)	530
(-)-Epicatechin gallate (ECG$^{\bullet}$/ECG)	550
(-)-Epicatechin (EC$^{\bullet}$/EC)	570
Rutin (R$^{\bullet}$/RH)	600
Kaempferol (K$^{\bullet}$/KH)	750

Oxidative damage inhibition in human cells through direct radical scavenging by plant polyphenol antioxidants has been reviewed by Es-Safi and Fraga (*211*, *212*). Similar to lipid peroxidation prevention, the polyphenol structural properties that result in the most effective radical scavenging activity are: 1) a catechol or a gallol group, 2) a C2-C3 double bond in the C ring and, 3) 3-hydroxyl and 4-oxo substituents in the C ring (*170*, *211*, *212*). Among all the polyphenol antioxidants tested in human cells, myricetin has all three structural components and appears to be the most effective radical scavenger (*369*). Quercetin and kaempferol have both a 4-oxo group and a C2-C3 double bond in the C ring, and are both very good ROS scavengers (*246*). Tea polyphenols such as EGCG and ECG do not have the C2-C3 double bond in ring C and are weaker radical scavengers, but the catechol and gallol groups on the B ring make them more potent radical scavengers compared to polyphenols without catechol groups (*366*).

A study by Wang *et al.* (*170*) in mouse cells provided detailed trends and structural comparisons of polyphenol antioxidant abilities lacking in human cellular studies. The ability of quercetin, myricetin, morin, gisetin, and kaempferol (0.1-10 μM) to prevent cell death by H_2O_2 challenge (1 mM for 24 h) in mouse macrophage-like cells (RAW 264.7) was compared to results of *in vitro* DPPH, $O_2^{\bullet-}$, and iron-generated $^{\bullet}OH$ scavenging assays to determine the effects of polyphenol structural differences on observed antioxidant activity. Catechol-containing quercetin, myricetin, and gisetin scavenged *in vitro* ROS more effectively in the DPPH assay (IC_{50} values of 8 - 12 μM) compared to kaempferol and morin (IC_{50} > 20.0 μM), polyphenol compounds without catechol groups. In contrast, kaempferol and morin showed significantly more effective cell death prevention (71% at 10 μM) and *in vitro* $O_2^{\bullet-}$ and $^{\bullet}OH$ scavenging abilities (IC_{50} ~0.5 μM) than myricetin and gisetin (no cellular activity, ~30% $O_2^{\bullet-}$ scavenging at 10 μM, and $^{\bullet}OH$ scavenging IC_{50} = 2.5-5 μM), whereas quercetin showed mixed results (IC_{50} ~10 μM for cell death prevention and IC_{50} values of ~8 and 0.5 μM for $O_2^{\bullet-}$ and $^{\bullet}OH$ scavenging, respectively) (*170*). Although the antioxidant effects of these polyphenols were attributed to ROS scavenging, overlapping mechanisms may have been examined in the cellular studies: the $O_2^{\bullet-}$ and $^{\bullet}OH$ scavenging results likely involved antioxidant enzyme regulation effects and iron chelation ability, respectively, as well as ROS scavenging. In addition, polyphenol bioavailability differences were not taken into account in this study.

Several studies also reported that supplementation with EGCG and polyphenol-containing *Geranium sanguineum* extracts (0.1-100 μM) increased resistance of human erythrocytes to H_2O_2-mediated cytotoxicity in a dose-responsive manner by an ROS scavenging mechanism (*370*), although cell death upon H_2O_2 challenge in mammalian cells, as in *E. coli*, is primarily due to an iron-mediated pathway (*176*, *371*). The presence of possible overlapping antioxidant mechanisms, especially in cellular studies, may lead to overestimation of polyphenol ROS scavenging abilities if other mechanisms are not also considered.

Owen *et al.* (*372*) used thiobarbituric acid (TBA) and DPPH scavenging assays to demonstrate that Melanesian polyphenol-rich plant extracts inhibit oxidized-LDL-induced cell death in human endothelial cells. The most abundant polyphenol in these extracts, quercetin, was believed responsible for the majority of radical scavenging ability. Owen and coworkers also reported that these extracts significantly inhibited Cu^+-induced lipid peroxidation in a dose-dependent manner. Because the results from the Cu^+-induced lipid peroxidation studies did not correlate well with the TBA and DPPH scavenging results, the authors concluded that lipid peroxidation inhibition by plant extracts was not related to ROS scavenging (*372*), but did not mention copper chelation as a possible alternate mechanism.

In addition to catechol-containing polyphenol antioxidants, silymarin (Figure 3), a flavanone with a 3-hydroxyl group on the C ring was also found to have an extremely high ROS scavenging ability. Silymarin studies in human cells primarily focused on enzyme regulation, but Chlopcikova *et al.* (*373*) used the DPPH scavenging assay to compare antioxidant activities of quercetin and silymarin (20-100 μM) in rat heart microsomes. They concluded that silymarin inhibits doxorubicin-mediated damage primary by radical scavenging, but several studies also reported that silymarin also chelates iron and copper (*234*, *257*, *258*, *373*). Additional studies used the total oxyradical scavenging capacity (ACAP) assay to determine the ROS scavenging ability of silymarin, and results showed that physiologically relevant doses of silymarin (100 mg/kg/d) protected rat liver mitochondria and aged rat brain from lipid peroxidation (*89*, *218*, *374*).

The ROS scavenging ability of polyphenol antioxidants is well established using many different methods and is a major mechanism for their antioxidant activity in human and other mammalian cells. However, polyphenol ROS scavenging abilities may be overestimated due to inadvertent testing of several mechanisms in a single assay. Many studies use iron or copper to generate ROS or lipid peroxidation, and because polyphenol compounds can chelate metal

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

ions as well as scavenge ROS, antioxidant activity results may vary due to this overlap between metal chelation and ROS scavenging mechanisms. Thus, using ROS scavenging assays without added metal ion and performing experiments in metal-reduced or metal-free systems, as well as comparing results from several complementary ROS scavenging assays, are required to establish accurate ROS scavenging capacities for polyphenol compounds.

7.2. Polyphenol Enzyme Regulation in Human Cells

Polyphenol antioxidants can affect the activity of many mammalian enzyme systems *in vitro* and *in vivo* (*11*, *52*, *230*, *237*, *304*). It is estimated that 75,000 enzymes are present in humans and that more than 100 of these enzymes are involved in ROS regulation or defense (*375*). Enzyme systems in human cells are significantly more complex than in *E. coli*; however, the basic enzymatic pathways can be similar. In this section, polyphenol regulation of ROS-related enzymes will be discussed, as well as the similarities and differences of polyphenol enzyme regulation in *E. coli* and human cell studies.

Protein kinase C (PKC) is a multifunctional serine- and threonine-phosphorylating enzyme involved in tumor promotion, secretory processes, and inflammation (*172*, *376*). Several studies have shown that quercetin, gisetin, and EGCG (50-250 μM) activate PKC and increase cell survival (*172*, *377*). Levites *et al.* (*172*) showed that EGCG reactivated PKC and extracellular signal-regulated kinases (ERK ½) damaged by 6-hydroxydopamine (6-OHDA) in human neuronal cell cultures. The Parkinsonism-inducing neurotoxin, 6-OHDA, induced neuronal death after 24 h exposure, and pretreatment with EGCG (50 μM) increased the cell survival rate by 80% (*172*). Western blot studies also showed that EGCG pretreatment (0.1-10 μM) increased PKC and ERK ½ levels in a dose-dependent manner and that EGCG neuroprotection was inhibited by adding a PKC inhibitor GF (109203X), indicating that EGCG protected against 6-OHDA-induced oxidative damage by reactivating PKC expression (*172*).

Lin *et al.* (*330*) also showed that quercetin supplementation (10-80 μM) decreased matrix metalloproteinase 9 (MMP-9) expression induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in a human breast carcinoma cell line (MCF-7). Western blot results demonstrated that TPA-induced MMP-9 expression was also inhibited by a PKC inhibitor (GF 109203X), an AP-1 inhibitor (curcumin), and an ERK inhibitor (PD98059), similar to effects observed upon quercetin treatment. In contrast, MMP-9 levels were not reduced by addition of an NF-κB inhibitor (PDTC), indicating that quercetin lowers MM9-1 expression by reducing PKC, AP-1, and ERK expression, but not NF-κB expression. Other polyphenol compounds were also tested, including rutin, quercitrin, luteolin, and fisetin (Figure 3), to elucidate substituent effects on MMP-9 expression (*330*). From this comparison, the catechol group on the B ring was found to be the most important substituent for decreasing MMP-9 levels, along with methylation of the 3′- and 4′-hydroxyl substituents. Hydroxyl substituents at the 3, 5, and 7 positions also decreased enzyme expression, with the presence of a 3-hydroxyl group having the greatest effect (*330*). This study is consistent with animal studies that indicated quercetin might reduce oxidative stress by inhibiting PKC/ERK

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

expression (*236*). Rutin and quercitrin treatment did not affect MMP-9 expression (*330*), demonstrating the importance of polyphenol cellular bioavailability. The sugar moiety at the 3 position in rutin and quercitrin likely prevented large concentrations of these polyphenols from entering cells, consistent with reports that carbohydrate substituents must be cleaved from polyphenol compounds to allow diffusion through the cell membrane (*378*).

Asano *et al.* (*379*) showed that quercetin (20-100 μM) stimulates Cl^- secretion by activating an energy step across the basolateral membrane through the $Na^+/K^+/2Cl^-$ cotransporter protein (NKCC1) in the human airway epithelium. NKCC1 expression at the membrane surface was measured by bicinchoninic acid (BCA) protein assays and NKCC1 activity was measured using $^{86}Rb^+$ uptake as a tracer for K^+ (*379*). To clarify the mechanism of activation, a PKA inhibitor (PKI 14-22 amide), a PKC inhibitor (Go 6983), and a Ca^{2+} chelating agent (BAPTA-AM) were also tested in this study. Only the PTK inhibitor significantly inhibited quercetin-stimulated Cl^- uptake, indicating that quercetin effected Cl^- secretion *via* a PTK-mediated pathway (*379*). In addition, an epidermal growth factor receptor (EGFR) kinase inhibitor (AG-1478) also inhibited quercetin-stimulated Cl^- secretion, suggesting that quercetin activated EGFR tyrosine kinase to stimulate translocation of an NKCC1-activating factor to the membrane. This study demonstrated the effects of quercetin on human epithelial cells, and suggested that this polyphenol may protect against bacterial and viral infection by helping to maintain appropriate Cl^- ion concentrations in airway surface fluid (*379*).

EGCG-mediated protection of human endothelial cells by enzyme regulation was examined by Zheng and coworkers (*380*). In this study, cells were pretreated with EGCG (30 μM for 4 h) and then exposed to tumor necrosis factor-α (TNF-α), a proinflammatory cytokine contributing to endothelial inflammation. EGCG prevented endothelial cell death by interacting with heme oxygenase-1 (HO-1), an enzyme with potent antioxidant and anti-apoptotic abilities, and increasing its expression by 2-fold as well as reducing AP-1 binding to DNA (*380*). Since AP-1 is a transcription factor activated by TNF-α during endothelial inflammation and is dependent on functional HO-1, Zheng and others concluded that HO-1 expression induced by polyphenols inhibited TNF-α-stimulated AP-1 activation associated with endothelial inflammation (*380*, *381*). In a related study, Wu and coworkers proposed that EGCG (25-100 μM) induced HO-1 expression *via* a phosphatidylinositol 3-kinase (PI3K)/Akt and ERK pathway (*382*).

Polyphenol-rich plant extracts from pine bark (0.05 mg/mL) were also found to prevent cell apoptosis in human myoblasts under H_2O_2-induced oxidative stress (1 mM for 1 h) (*383*). Western blot and immunofluorescence assays showed that these extracts increased levels of ATP synthase and calpain, a Ca^{2+}-dependent protease that induces cell apoptosis, to reduce oxidative DNA damage and increase cell survival by ~20%. Similar increases in ATP synthase and calpain levels were also reported upon EGCG treatment (*383*, *384*). Nicholson and coworkers (*385*) also showed pretreated polyphenol antioxidants such as resveratrol, EGC, and quercetin (0.1-1 μM, 24 h) in human umbilical vein endothelial cells (HUVECs) increased eNOs and VEGF mRNA expression and inhibited H_2O_2-induced ET-1 mRNA expression by ~45% (*385*, *386*). This study was unusual for enzyme

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

activation studies because the polyphenol concentrations tested are in the range of dietary polyphenol concentrations found in human plasma, although the authors mentioned that polyphenol compounds may have been metabolized after 24 h, so it was not clear whether the observed effects were due to the supplemented polyphenols or their metabolites. In addition, a review by Mladenak (*235*) discussed the ability of polyphenol antioxidants (1-10 μM) to suppress the activity of ROS-generating enzymes, such as xanthine oxidase, NADPH oxidase, and lipoxygenases, by 2-3 fold and indirectly reduce cellular oxidative stress.

Polyphenol antioxidants can modulate enzyme regulation by directly interacting with proteins and causing structural alterations that increase or decrease enzyme activity. These changes in enzyme activity can either alter cell signaling to inhibit cell death or reduce oxidative stress by indirect ROS scavenging (*235*, *379*). Many studies of enzyme regulation by polyphenols examine only one specific enzymatic pathway to demonstrate cellular efficacy, resulting in fragmented information about this cellular antioxidant mechanism. In addition, levels of several antioxidant enzymes are regulated by ROS and metal concentrations, including glutathione peroxidase (GSHPx), catalase, and SOD (*174*, *387*). Thus, strong correlations between ROS and/or metal concentrations and enzyme levels may confound enzyme regulation studies and lead to inconsistent results, since it is difficult to separate direct and indirect polyphenol effects (*46*, *122*). In addition, enzymatic assays are typically performed at only one or two polyphenol concentrations and often use polyphenol concentrations higher than physiologically relevant polyphenol concentrations (2-1000 μM vs. ~10 μM) (*52*, *385*), although Nicholson and coworkers found that polyphenols effectively regulated enzyme mRNA levels at lower polyphenol concentrations (0.1-1 μM) (*384*, *386*). Polyphenol antioxidants can modulate enzyme activities, but, as seen for ROS scavenging, the primary mechanisms for polyphenol antioxidant activity are interrelated. Because of this, comparing polyphenol effects on enzyme function with those of specific enzyme inhibitors is the most accurate method to establish direct enzymatic effects (*113*, *331*, *379*, *388–390*).

7.3. Polyphenol-Metal Chelation in Human Cells

As mentioned previously, the majority of cellular $^{\bullet}$OH is generated by the iron-mediated Fenton reaction (*66*), and copper, chromium, cobalt, and other redox active metals can also undergo Fenton-like reactions (*66*, *244*). In fact, iron-mediated DNA damage by $^{\bullet}$OH is the primary mechanism for human cell death under oxidative stress conditions (*11*, *66*, *202*). Polyphenol antioxidants strongly chelate metal ions, and this binding can prevent iron-mediated $^{\bullet}$OH formation and the resulting oxidative stress (*29*).

In cellular oxidative challenge studies, addition of different ROS precusors generate ROS through different cellular pathways that enable researchers to distinguish cellular polyphenol antioxidant mechanisms. For example, Hanneken and coworkers (*391*) found that both H_2O_2 and *tert*-butylhydroperoxide (*t*-BuOOH) challenge of human epithelial cells resulted in iron-generated hydroxyl radical damage (Reaction 1), but H_2O_2-promoted cell death was prevented by both ROS scavengers and metal chelators, whereas *t*-BuOOH-promoted cell death was prevented by only metal chelators (*391*, *392*). Unlike H_2O_2, *t*-BuOOH cannot directly oxidize polyphenols (*391*, *392*), so comparing the differences between polyphenol prevention of cell death upon H_2O_2 and *t*-BuOOH challenge provides a potential method for distinguishing between polyphenol ROS scavenging and iron chelation mechanisms. Hanneken *et al.* (*246*) used both H_2O_2 (250 μM) and *t*-BuOOH (120 μM for 24 h) challenge to examine the antioxidant effects of fisetin, luteolin, quercetin, eriodictyol, baicalein, galangin, and EGCG (5-50 μM) in human retinal pigment epithelial cells (ARPE-19) and found that these polyphenol compounds prevented 100% of cell death from both types of oxidative challenge. In this study, however, the authors did not discuss the differences in polyphenol antioxidant mechanisms tested by each oxidant challenge and reported that the primary antioxidant mechanism was ROS scavenging.

Sestili *et al.* (*246*, *392*) showed that polyphenol treatment (0.1-100 μM) prevented DNA damage and cell death in human histiocytic lymphoma cells (U937) upon *t*-BuOOH challenge (3 mM, 30 min) with very low IC_{50} values (0.006-2.73 and 0.02-12 μM, respectively). Since *t*-BuOOH-mediated cell death is prevented by iron chelators and not ROS scavengers (*246*), these studies were designed to specifically test the iron-binding mechanism for polyphenol antioxidant activity. In these studies, polyphenol prevention of cell death was comparable to that of *o*-phenanthroline, a well-known iron chelator (*392*, *393*). Polyphenols with iron binding catechol groups inhibited DNA damage and cell death more effectively than polyphenol compounds without iron-binding groups, and cellular efficacy correlated with polyphenol hydrophobicity (*394*). Based on these results, the authors concluded that polyphenol compounds inhibited DNA damage and cell death at biologically relevant concentrations primarily through iron binding rather than ROS scavenging (*246*, *392*).

Boadi *et al.* (*395*) compared the ability of quercetin and genistein (50 μM, Figure 3) to prevent of Cu^{2+}- and Fe^{2+}-induced (50 μM) lipid peroxidation upon H_2O_2 challenge (0.01 mM for 24 h) in human cells (U937). Cells supplemented with the polyphenol antioxidants (10 or 20 μM for 24 h) showed an ~80% decrease in lipid peroxidation, as measured by the TBARS assay, with no significant differences in results for the two compounds or between the two metal ions. In previous work from the same group, quercetin was found to have stronger metal chelation ability than genistein due to the additional hydroxyl substituent that forms a catechol group on the B ring (*345*). Although the similar antioxidant abilities of quercetin and genistein led the authors to exclude a metal chelation mechanism (*395*), the greater hydrophobicity of genistein compared to quercetin may result in higher cellular bioavailability and larger antioxidant effects for genistein.

Polyphenol-rich plant extracts from *Vicia sativa* enabled 2-fold greater cell proliferation in human THP-1 leukemic cells and 2-3 fold greater Cu^{2+} and Fe^{2+} chelation ability *in vitro* compared to soybean extracts. Magias and coworkers (*396*) compared the ability of these extracts to increase cell proliferation relative to the well-studied antioxidant butylated hydroxytoluene (BHT) and correlated these results with *in vitro* metal binding (using spectrophotometry and metal reducing assays) and ROS scavenging (using DPPH assays). From these correlations, the authors concluded that metal-binding ability, not ROS scavenging, was the

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

primary polyphenol antioxidant mechanism and that *Vicia sativa* and soybean extracts exhibited different antioxidant abilities due to differences in polyphenol content (*396*). This study carefully compared metal binding and ROS scavenging mechanisms for polyphenol antioxidant activity, and is one of the few to clearly demonstrate polyphenol-metal chelation as a primary cellular mechanism.

In studies by Thephinlap and coworkers (*397*, *398*), erythrocytes from β-thalassemia patients were challenged with H_2O_2 (1 mM) with and without treatment with green tea extracts (0-5 g/mL) and EGCG or EGC (1-1000 μM) for up to 8 h. Upon polyphenol or extract treatment, cellular oxidative stress measured by the TEAC assay was reduced by 35%. *In vitro* polyphenol radical scavenging (using malonyldialdehyde, TBA, and TEAC assays) and non-transferrin-bound iron (NTBI) chelation abilities were also measured for mechanistic comparisons. NTBI chelation results indicated a strong correlation between polyphenol iron-binding abilities and reduction in cellular labile iron concentrations in a time- and dose-dependent manner, and polyphenols with strongly-iron-binding gallol groups contributed most to reducing cellular iron concentrations (*366*, *397*, *399*). Since cellular iron concentrations were lowered by ~40% after pre-treatment with EGCG and EGC, the authors concluded that polyphenol-iron binding was the major mechanism for oxidative stress reduction in β-thalassemic erythrocytes and suggested that EGCG and ECG may be natural iron chelators to help treat iron overload in β-thalassemia patients (*398*). Later studies with polyphenol-treated β-thalassemic mice further supported this hypothesis (*399*).

In addition to Fe^{2+} and Cu^{2+}, Quesada *et al.* (*400*) demonstrated that catechin, epicatechin, ECG, EGCG, and other polyphenol compounds (10-100 μM) chelate cellular Zn^{2+} to modulate zinc homeostasis in HepG2 cells. Using spectrophotometric methods, polyphenol compounds were found to bind Zn^{2+} with 2:1 stoichiometry (*29*). In cells, EGCG and related polyphenol antioxidants bound to zinc and lowered the expression of metallothionein (MT), a Zn^{2+}-binding protein that modulates zinc homeostasis, by up to 60% and with greater efficacy than the known zinc chelator *N*,*N*,*N*',*N*'-tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN, 15% at 100 μM) (*400*). Quesada and coworkers also measured total intracellular zinc concentrations and found that zinc concentrations in polyphenol-treated cells remained constant, similar to zinc levels of cells grown in zinc-depleted medium, whereas zinc concentrations steadily increased in untreated cells. Thus, the authors concluded that polyphenol compounds bound to Zn^{2+} in medium (~5 μM), and that these Zn^{2+}-polyphenol complexes were not able to enter cells through metal ion transporters, preventing zinc overload and lowering MT expression (*400*).

Lowering of *in vivo* iron levels has also been observed upon polyphenol supplementation. Oral consumption of polyphenol-rich grape seed extract for 90 days in rats lowered serum iron levels by 14-17% compared to non-treated controls (*401*). In humans, dietary intake of polyphenols such as tannic acid, gallic acid, and oregano polyphenols (5-100 mg) inhibited iron absorption by 20 to 88% in a dose-responsive manner, and this inhibition strongly correlated with the content of iron-binding gallol groups in the polyphenol supplements (*402*). Similar to the cellular studies with zinc, these effects were believed due to metal uptake inhibition resulting from polyphenol-metal binding, although no

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

direct evidence of polyphenol-iron binding and its effects on iron absorption was presented in these studies.

Accumulating evidence suggests that metal chelation is a major mechanism for polyphenol reduction of oxidative stress in human cells. Given the strong metal chelating abilities of polyphenol compounds and the extremely short lifetimes of many radical species, especially hydroxyl radical (half life ~10^{-9} s) (*198*), preventing reactive radical generation by polyphenol-iron binding may more effectively prevent oxidative stress compared to radical scavenging. Metal chelation, however, is the least-studied of the three major antioxidant mechanisms, and more work is required to carefully elucidate the effects of cellular metal chelation by polyphenol antioxidants. Several studies have excluded the possibility of metal-binding mechanisms due to the poor correlation between *in vitro* and cell study results (*126*, *365*, *385*, *395*), but differences in polyphenol bioavailability also must be considered when comparing *in vitro* and cellular results in studies of any mechanism.

In the polyphenol antioxidant literature, the complex interrelationships between ROS scavenging, enzyme regulation, and metal binding mechanisms of activity can lead to complex and conflicting results. Further complications can arise because polyphenol compounds are partially ionized in the physiological pH range and have different hydrophobicities and bioavailabilities due to structural differences (*29*, *394*). These differences can lead to significant changes in cellular uptake and can also significantly alter antioxidant mechanisms. For example, polyphenols with higher pK_a values will be more hydrophobic and potentially cross cell membrane in greater concentrations, but their lower ionization at physiological pH may also inhibit polyphenol-metal binding. Thus, to advance mechanistic studies of polyphenol effects, cellular uptake must be established and compared for a wide variety of polyphenols. To realize the significant promise of polyphenol antioxidants to prevent or treat a wide range of diseases caused by oxidative stress, understanding polyphenol antioxidant mechanisms is required to identify the most effective antioxidants for animal and clinical studies.

8. Conclusions and Future Directions

The large number of experimental methods to test antioxidant activity and the difficulty in selecting methods that test only a single mechanism are testaments to the wide range and complexity of potential mechanisms for polyphenol antioxidant behavior. Many of the reported ROS scavenging measurements *in vitro* and in cells are conducted in the presence of metal ions to induce oxidative stress, making it unclear whether the results of these measurements reflect the ability of polyphenol antioxidants to directly scavenge ROS or to bind metals and inhibit ROS formation. In addition, the commonly accepted belief that ROS scavenging is the primary antioxidant mechanism for polyphenols has limited research into other possible mechanisms of activity. In general, when the effective polyphenol concentrations for ROS scavenging, enzyme regulation, and metal-chelating mechanisms are compared in *E. coli* and human cells, polyphenol-metal chelation mechanisms generally result in

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

antioxidant activity at low micromolar concentrations (IC_{50} values of ~1-30 μM), whereas ROS scavenging and enzyme regulation mechanisms typically have effective concentrations in high micromolar to low millimolar ranges (IC_{50} values of ~10-1000 and ~10-10,000 μM, respectively). These effective concentrations indicate that metal chelation should be examined closely as a polyphenol antioxidant mechanism in addition to ROS scavenging and enzyme regulation, although it is likely that most polyphenols are active through different combinations of all three mechanisms.

Polyphenol research is a popular field due to their potential for improving human health, and researchers have expended much effort to elucidate polyphenol antioxidant mechanisms. As is clear from the results of *in vitro*, *E. coli*, and human cell studies, polyphenol antioxidant mechanisms are complex, interrelated, and often difficult to determine with certainty with many experimental methods. This complexity results in inconsistencies between *in vitro* and cellular results as well as difficulties in comparing results among separate studies. In addition to cellular effects, determining polyphenol antioxidant activity in humans also depends on understanding polyphenol intake, absorption, and metabolism; however, these properties have been examined for only a few of the thousands of polyphenol compounds (*12*, *403*). Methods to increase polyphenol solubility and bioavailability, such as addition of water-soluble and pK_a-altering substituents, may enhance tissue uptake and antioxidant efficacy and are currently being investigated (*25*, *154*, *160*).

8.1. Polyphenol Bioavailability and Metabolism

Polyphenols can be present as polymers or in glycosylated forms in food before ingestion. These hydrophilic glycosylated polyphenols are normally too large or polar to pass through the small-intestinal membrane and must be hydrolyzed by intestinal enzymes or colonic microflora before absorption (*49*, *159*). Two possible mechanisms involving two intestinal enzymes can hydrolyze polyphenols into their more absorbable aglycone forms (Figure 14). The first enzyme, lactase phloridizin hydrolase (LPH), is present in the brush-border of small intestine epithelial cells (*159*). LPH will either hydrolyze lactose substituents or deglycosylate more hydrophobic substituents on the polyphenol to increase lipophilicity, and the resulting aglycone polyphenols then pass through the intestinal membrane by passive diffusion (Figure 15) (*378*). The second enzyme, cytosolic β-glucosidase (CBG) is found in a wide variety of tissues, especially in liver and intestinal epithelial cells, and catalyzes the hydrolysis of polyphenol glycosides to improve bioavailability (*404*). Polyphenols that are not absorbed in the stomach or the small intestine will be carried to the colon (Figure 15). Polyphenols metabolized in the liver and excreted into the bile will also be excreted into the small intestine and enter the colon. The colon contains ~10^{12} microflora per cubic centimeter (*25*, *47*, *159*), and these microflora catalyze the breakdown of the polyphenol itself, not only hydrolyzing polyphenol glycosides, but also degrading polyphenols into phenolic acids. For example, human colonic microflora degrade procyanidin polymers into phenolic acids that can be absorbed through the colon (*405*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

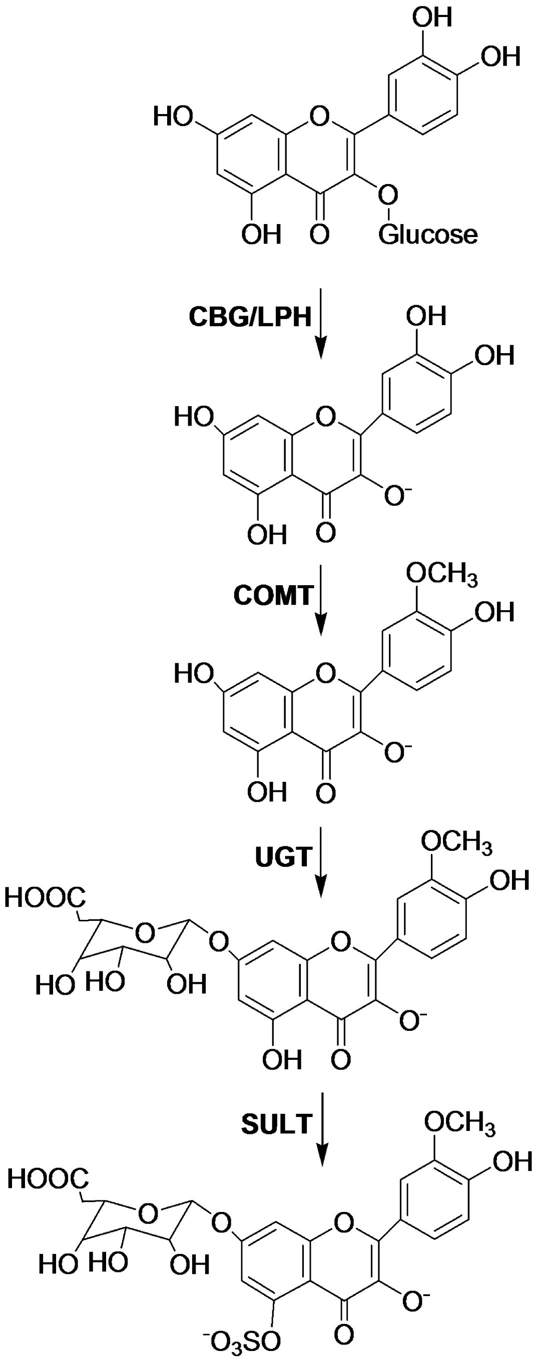

OH
OH
HO
O
OH
O
O
Glucose
CBG/LPH
OH
OH
HO
O
O⁻
OH
O
COMT
OCH3
OH
HO
O
O⁻
OH
O
UGT
OCH3
OH
HOOC
O
O
O
HO
HO
OH
O⁻
OH
O
SULT
OCH3
OH
HOOC
O
O
O
HO
HO
OH
O⁻
⁻O3SO
O

Figure 14. Polyphenol metabolism using quercetin as an example (159, 404).

Simple aglycone polyphenols pass through the intestinal membrane but can still have poor bioavailability due to their low solubility (20 µg/mL in water) and slow dissolution rates (*47*, *369*). Therefore, polyphenols are modified by methylation, sulfonation, glucuronidation or a combination of these by catechol-O-methyltransferase (COMT), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), and other enzymes in the intestine or liver to increase uptake (Figure 14) (*41*, *74*). Only a small portion of these conjugated polyphenol metabolites enter the bloodstream; most are excreted into the intestinal lumen (*406*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

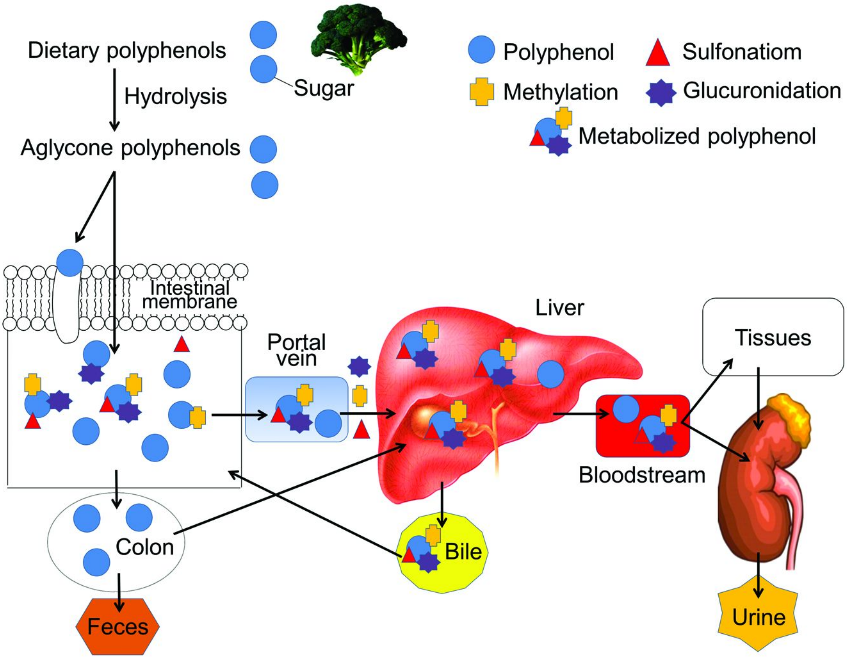

Dietary polyphenols
Hydrolysis
Sugar
Aglycone polyphenols
Polyphenol
Sulfonatiom
Methylation
Glucuronidation
Metabolized polyphenol
Intestinal membrane
Portal vein
Liver
Tissues
Bloodstream
Colon
Bile
Feces
Urine

Figure 15. Possible metabolic pathways for polyphenol compounds in humans.

COMT generally catalyzes methylation of 3′- and 4′-hydroxyl substituents on the B ring of catechol- or gallol-containing polyphenols, but different polyphenol structures will determine which hydroxyl groups on the polyphenol ring are methylated (*56*, *404*). In contrast, cytochrome P450 demethylates flavonols at the 4′ position and not at the 3′ position, so the specificity of catechol or gallol methylation is controlled by both COMT methylation and cytochrome P450 demethylation (*45*, *164*). UGT catalyzes polyphenol glucuronidation on 5- and 7-hydroxyl substituents to form glucuronic-acid-conjugated polyphenols in the intestine, liver, and kidney (*407*). SULTs comprise a third group of cytosolic enzymes found in the liver, colon, and other organs that catalyze the sulfate conjugation of polyphenol compounds, leading to changes in thermal stability and bioavailability (Figure 14) (*403*). Although it was suggested that sulfonation typically occurred at the same conjugation sites as glucuronidation, studies of quercetin metabolism have shown that sulfonation occurs on the 3′-hydroxyl substituent to form quercetin-3′-sulfate (Figure 14) (*231*). In addition to increasing antioxidant activity, these conjugation processes reduce potential polyphenol toxicity by increasing solubility and molecular weight to facilitate excretion in the urine or bile (*159*). Further details of polyphenol metabolic processes are being actively investigated.

Polyphenol metabolites also contribute to polyphenol antioxidant activity due to their greater bioavailability; in fact, some metabolites have better antioxidant activity compared to unmetabolized polyphenols (*39*, *41*, *42*, *52*, *121*). Understanding how dietary polyphenols can prevent or treat human diseases and determining appropriate polyphenol doses will require accurate measurements of polyphenol uptake in specific organs and tissues, measurements that may be more important than determining polyphenol plasma concentrations (*241*, *408*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

8.2. Future Research Directions

Polyphenol compounds have multiple biological effects and hold great promise for prevention of chronic diseases such as cancer, stroke, and neurodegenerative and cardiovascular diseases (*7–9*, *409*, *410*); however, most cellular and animal studies have been conducted on short timescales (hours to days). Additional long-term studies (weeks to months) are needed to determine polyphenol biological effects relevant to chronic disease development. In addition to elucidating antioxidant mechansims of polpyhenol antioxidants individually, antioxidant effects of multiple polpyhenol compounds in combination, such as in plant extracts or whole foods, must also be investigated. Since polpyhenol antioxidant mechanisms are highly dependent upon structural differences, mixtures of polpyhenol compounds may act through multiple mechanisms even more than single compounds. In fact, several studies examining combined polyphenol treatment *in vitro* and in animal studies showed greater antioxidant effects than single-polyphenol treatment, indicating possible complimentary effects on polpyhenol activity (*151*, *345*).

Most important to developing a clear understanding of cellular polyphenol antioxidant activity is the careful selection of experimental methods capable of distinguishing between distinct antioxidant mechanisms. Many literature reports present inconsistent interpretations of experimental data, mainly due to a persistent focus on polyphenols as direct ROS scavengers.ï¿½ Even though ROS scavenging is certainly one of the major polyphenol antioxidant mechanisms, focusing on this mechanism to the exclusion of other possible mechanisms, such as enzyme regulation and metal chelation, slows research progress in this field.ï¿½ Ideally, studies should independently test several polyphenol antioxidant mechanisms and take into account differences in polyphenol bioavailability when analyzing and comparing results.ï¿½ The results of such studies will enable researchers to better understand polyphenol biological effects, leading to the development of predictive models for polyphenol antioxidant activity.ï¿½ Such models will help identify the most effective polyphenol compounds, out of the thousands known, for animal and human studies of disease prevention and treatment.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

9. Abbreviation List

4CL	4-coumaroyl–CoA ligase
5-LOX	5-lypooxygenase
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACAP	total oxyradical scavenging capacity
AD	Alzheimer's disease
AP-1	activator protein
BCA	bicinchoninic acid
BP	bisphenol
BHT	butylated hydroxytoluene
CAT	catalase
CBG	cytosolic β-lucosidase
CHI	chalcone isomerase
CHS	chalcone synthase
CK	creatine kinase
COMT	catechol-O-methyltransferase
COX-2	cyclooxygenase-2
CVD	cardiovascular disease
Cyt-P450	cytochrome P450
DAHP	2-keto-3-deoxyarabina-heptulosonate-7-phosphate
DEA	diethylamine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	dithiothreitol
E-4-P	erytheose-4-phosphate
EC_{50}	effective concentration at 50% activity
ECG	(-)-epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGCG	(-)-epigallolcatechin-3-gallate
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Endo IV	endonuclease IV
eNOS	endothelial nitric oxide synthase
EPR	electron paramagnetic resonance
ERK 1/2	extracellular signal-regulated kinases
FRAP	ferric reducing ability assay
GC	gas chromatography
Glut-1	glucose transporter-1
GPx	glutathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
GorA	glutathione reductase
GrxA	glutaredoxin A
ETC	electron transport chain
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HIF1-α	hypoxia-inducible factor 1-h

HO-1	haemoxygenase-1
HOONO	peroxynitrous acid
HPLC	high-performance liquid chromatography
H/R	hypoxia-reoxygenation
HUVECs	human umbilical vein endothelial cells
IC_{50}	concentration at 50% inhibition
ICAM	intercellular adhesion molecule
IL	interleukin
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
L-NAME	L-N^G-nitroarginine methyl ester
LPH	lactase phloridizin hydrolase
MA	malonaldehyde
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein
MMP	matrix metalloproteinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MT	metallothionein
NADPH	nicotinamide adenine dinucleotide phosphate
Nb	neuroglobin
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NKCC1	$Na^+/K^+/2Cl^-$ cotransporter
$NO^•$	nitric oxide
NOS	nitric oxide synthase
NTBI	non-transferrin-bound iron
$O_2^{•-}$	superoxide radical
$^•OH$	hydroxyl radical
6-OHDA	6-hydroxydopamine
8-oxo-dG	8-oxo-7,8-dihydro-2′-deoxyguanosine
$ONOO^-$	peroxynitrite anion
P53	tumor protein 53
PI3K	phosphatidylinositol 3-kinase
PAL	phenylalanine ammonia lyase
PD	Parkinson’s disease
PEP	phosphoenolpyruvate
$PhenO^•$	phenoxy radical
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PTK	protein tyrosine kinase
PYC	Pycnogenol
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
STAT-1	signal transducer and activator of transcription
SULT	sulfotransferases

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

TBA	thiobarbituric acid assay
t-BuOOH	*tert*-butylhydroperoxide
TCA	tricarboxylic acid
TEAC	Trolox-equivalent antioxidant capacity assay
TNF- α	tumor necrosis factor- α
TPA	12-O-tetradecanoylphorbol-13-acetate
TPEN	N,N,N′,N′-tetrakis(2-pyridyl-methyl)ethylenediamine
TRAMP	transgenic adenocarcinoma of the mouse prostate
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UGT	UDP-glucuronosyltransferases
VCAM	vascular cell adhesion protein
VEGF	vascular endothelial growth factor
XO	xanthine oxidase

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

References

1. Kant, A. K.; Leitzmann, M. F.; Park, Y.; Hollenbeck, A.; Schatzkin, A. *J. Nutr.* **2009**, *139*, 1374–1380.
2. McKay, D. L.; Chen, C. Y.; Yeum, K. J.; Matthan, N. R.; Lichtenstein, A. H.; Blumberg, J. B. *Nutr. J.* **2010**, *9*, 21–30.
3. Ahmed, S. *Arthritis Res. Ther.* **2010**, *12*, 208–216.
4. Dreger, H.; Lorenz, M.; Kehrer, A.; Baumann, G.; Stangl, K.; Stangl, V. *Exp. Biol. Med.* **2008**, *233*, 427–433.
5. Barone, E.; Calabrese, V.; Mancuso, C. *Biogerontology* **2009**, *10*, 97–108.
6. Guo, W.; Kong, E.; Meydani, M. *Nutr. Cancer* **2009**, *61*, 807–810.
7. Halliwell, B. *Biochem. J.* **2007**, *401*, 1–11.
8. Mena, S.; Ortega, A.; Estrela, J. M. *Mutat. Res.* **2009**, *674*, 36–44.
9. Andreassi, M. G. *J. Mol. Med.* **2008**, *86*, 1033–1043.
10. Barzilai, A.; Yamamoto, K. *DNA Repair* **2004**, *3*, 1109–1115.
11. Blokhina, O.; Virolainen, E.; Fagerstedt, K. V. *Ann. Bot.* **2003**, *91*, 179–194.
12. Manach, C.; Mazur, A.; Scalbert, A. *Curr. Opin. Lipidol.* **2005**, *16*, 77–84.
13. Kowluru, R. A.; Kanwar, M. *Nutr. Metab.* **2007**, *4*, 8–15.
14. Rastmanesh, R. *Chem. Biol. Interact.* **2010**, *189*, 1–8.
15. Mosovska, S.; Mikulasova, M.; Brindzova, L.; Valik, L.; Mikusova, L. *Food Chem. Toxicol.* **2010**, *48*, 1483–1487.
16. SciFinder search April 21, 2011, using the term "polyphenol", refined by year (2001−current) and reviews only (duplicate entries removed).
17. SciFinder search on April 21, 2011, using the terms "polyphenol" and "antioxidant", refined by year (2001−current) and reviews only (duplicate entries removed).
18. Imlay, J. A.; Chin, S. M.; Linn, S. *Science* **1988**, *240*, 640–642.
19. Imlay, J. A.; Linn, S. *Science* **1988**, *240*, 1302–1309.
20. Hoffmann, M. E.; Mello-Filho, A. C.; Meneghini, R. *Biochim. Biophys. Acta* **1984**, *781*, 234–238.
21. Mello-Filho, A. C.; Meneghini, R. *Mutat. Res.* **1991**, *251*, 109–113.

22. Ksouri, R.; Megdiche, W.; Falleh, H.; Trabelsi, N.; Boulaaba, M.; Smaoui, A.; Abdelly, C. *C R Biol.* **2008**, *331*, 865–873.
23. Kusirisin, W.; Srichairatanakool, S.; Lerttrakarnnon, P.; Lailerd, N.; Suttajit, M.; Jaikang, C.; Chaiyasut, C. *Med. Chem.* **2009**, *5*, 139–147.
24. Obrenovich, M. E.; Nair, N. G.; Beyaz, A.; Aliev, G.; Reddy, V. P. *Rejuvenation Res.* **2010**, *13*, 631–643.
25. Scalbert, A.; Williamson, G. *J. Nutr.* **2000**, *130*, 2073S–2085S.
26. Das, D. K. *Methods Enzymol.* **1994**, *234*, 410–420.
27. Jaakola, L.; Maatta-Riihinen, K.; Karenlampi, S.; Hohtola, A. *Planta* **2004**, *218*, 721–728.
28. Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A. *Am. J. Clin. Nutr.* **2008**, *88*, 38–50.
29. Perron, N. R.; Brumaghim, J. L. *Cell. Biochem. Biophys.* **2009**, *53*, 75–100.
30. Song, W. O.; Chun, O. K. *J. Nutr.* **2008**, *138*, 1543S–1547S.
31. Heller, W. *Prog. Clin. Biol. Res.* **1986**, *213*, 25–42.
32. Schijlen, E. G.; de Vos, C. H.; Martens, S.; Jonker, H. H.; Rosin, F. M.; Molthoff, J. W.; Tikunov, Y. M.; Angenent, G. C.; van Tunen, A. J.; Bovy, A. G. *Plant Physiol.* **2007**, *144*, 1520–1530.
33. Nones, J.; Stipursky, J.; Costa, S. L.; Gomes, F. C. *Neurochem. Res.* **2010**, *35*, 955–966.
34. Slimestad, R.; Fossen, T.; Vagen, I. M. *J. Agric. Food Chem.* **2007**, *55*, 10067–10080.
35. Somerset, S. M.; Johannot, L. *Nutr. Cancer* **2008**, *60*, 442–449.
36. Khokhar, S.; Magnusdottir, S. G. *J. Agric. Food Chem.* **2002**, *50*, 565–570.
37. Arai, Y.; Watanabe, S.; Kimira, M.; Shimoi, K.; Mochizuki, R.; Kinae, N. *J. Nutr.* **2000**, *130*, 2243–2250.
38. Ovaskainen, M. L.; Torronen, R.; Koponen, J. M.; Sinkko, H.; Hellstrom, J.; Reinivuo, H.; Mattila, P. *J. Nutr.* **2008**, *138*, 562–566.
39. Jin, S.; Zhang, Q. Y.; Kang, X. M.; Wang, J. X.; Zhao, W. H. *Ann. Oncol.* **2010**, *21*, 263–268.
40. Sakamoto, T.; Horiguchi, H.; Oguma, E.; Kayama, F. *J. Nutr. Biochem.* **2010**, *21*, 856–864.
41. Cornish, K. M.; Williamson, G.; Sanderson, J. *Free Radical Biol. Med.* **2002**, *33*, 63–70.
42. Georgetti, S. R.; Vicentini, F. T.; Yokoyama, C. Y.; Borin, M. F.; Spadaro, A. C.; Fonseca, M. J. *J. Appl. Microbiol.* **2009**, *106*, 459–466.
43. Shiba, Y.; Kinoshita, T.; Chuman, H.; Taketani, Y.; Takeda, E.; Kato, Y.; Naito, M.; Kawabata, K.; Ishisaka, A.; Terao, J.; Kawai, Y. *Chem. Res. Toxicol.* **2008**, *21*, 1600–1609.
44. Hollman, P. C.; Bijsman, M. N.; van Gameren, Y.; Cnossen, E. P.; de Vries, J. H.; Katan, M. B. *Free Radical Res.* **1999**, *31*, 569–573.
45. Hollman, P. C.; van Trijp, J. M.; Mengelers, M. J.; de Vries, J. H.; Katan, M. B. *Cancer Lett.* **1997**, *114*, 139–140.
46. Chow, H. H.; Hakim, I. A.; Vining, D. R.; Crowell, J. A.; Cordova, C. A.; Chew, W. M.; Xu, M. J.; Hsu, C. H.; Ranger-Moore, J.; Alberts, D. S. *Cancer Epidemiol., Biomarkers Prev.* **2006**, *15*, 2473–2476.
47. Clifford, M. N. *Planta Med.* **2004**, *70*, 1103–1114.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

48. Kim, S.; Lee, M. J.; Hong, J.; Li, C.; Smith, T. J.; Yang, G. Y.; Seril, D. N.; Yang, C. S. *Nutr. Cancer* **2000**, *37*, 41–48.
49. Chen, L.; Lee, M. J.; Li, H.; Yang, C. S. *Drug Metab. Dispos.* **1997**, *25*, 1045–1050.
50. Manach, C.; Morand, C.; Gil-Izquierdo, A.; Bouteloup-Demange, C.; Remesy, C. *Eur. J. Clin. Nutr.* **2003**, *57*, 235–242.
51. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.
52. Alia, M.; Ramos, S.; Mateos, R.; Granado-Serrano, A. B.; Bravo, L.; Goya, L. *Toxicol. Appl. Pharmacol.* **2006**, *212*, 110–118.
53. Intra, J.; Kuo, S. M. *Chem.−Biol. Interact.* **2007**, *169*, 91–99.
54. King, R. E.; Kent, K. D.; Bomser, J. A. *Chem.−Biol. Interact.* **2005**, *151*, 143–149.
55. Borges, G.; Mullen, W.; Mullan, A.; Lean, M. E.; Roberts, S. A.; Crozier, A. *Mol. Nutr. Food Res.* **2010**, *54*, S268–277.
56. Carbonaro, M.; Grant, G.; Pusztai, A. *Eur. J. Nutr.* **2001**, *40*, 84–90.
57. Del Rio, D.; Borges, G.; Crozier, A. *Br. J. Nutr.* **2010**, *104*, S67–90.
58. Kushi, L. H.; Byers, T.; Doyle, C.; Bandera, E. V.; McCullough, M.; McTiernan, A.; Gansler, T.; Andrews, K. S.; Thun, M. J. *CA−Cancer J. Clin.* **2006**, *56*, 254–281.
59. Artinian, N. T.; Fletcher, G. F.; Mozaffarian, D.; Kris-Etherton, P.; Van Horn, L.; Lichtenstein, A. H.; Kumanyika, S.; Kraus, W. E.; Fleg, J. L.; Redeker, N. S.; Meininger, J. C.; Banks, J.; Stuart-Shor, E. M.; Fletcher, B. J.; Miller, T. D.; Hughes, S.; Braun, L. T.; Kopin, L. A.; Berra, K.; Hayman, L. L.; Ewing, L. J.; Ades, P. A.; Durstine, J. L.; Houston-Miller, N.; Burke, L. E. *Circulation* **2010**, *122*, 406–441.
60. Li, W.; Nie, S.; Xie, M.; Chen, Y.; Li, C.; Zhang, H. *J. Agric. Food Chem.* **2010**.
61. Wood, L. G.; Wark, P. A.; Garg, M. L. *Antioxid. Redox Signaling* **2010**, *13*, 1535–1548.
62. Ramprasath, V. R.; Jones, P. J. *Eur. J. Clin. Nutr.* **2010**, *64*, 660–668.
63. Epstein, J.; Sanderson, I. R.; Macdonald, T. T. *Br. J. Nutr.* **2010**, *103*, 1545–1557.
64. Sinha, D.; Roy, S.; Roy, M. *Food Chem. Toxicol.* **2010**, *48*, 1032–1039.
65. Harish, G.; Venkateshappa, C.; Mythri, R. B.; Dubey, S. K.; Mishra, K.; Singh, N.; Vali, S.; Bharath, M. M. *Bioorg. Med. Chem.* **2010**, *18*, 2631–2638.
66. Jomova, K.; Vondrakova, D.; Lawson, M.; Valko, M. *Mol. Cell. Biochem.* **2010**, *345*, 91–104.
67. Singh, R.; Akhtar, N.; Haqqi, T. M. *Life Sci.* **2010**, *86*, 907–918.
68. Visavadiya, N. P.; Soni, B.; Dalwadi, N.; Madamwar, D. *Drug Chem. Toxicol.* **2010**, *33*, 173–182.
69. Chen, Y.; Lu, N.; Ling, Y.; Wang, L.; You, Q.; Li, Z.; Guo, Q. *J. Pharmacol. Sci.* **2010**, *112*, 37–45.
70. Darshan, D.; Frazer, D. M.; Anderson, G. J. *Expert Rev. Mol. Med.* **2010**, *12*, e36.
71. Kaplan, S. A. *J. Urol.* **2005**, *173*, 914.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

72. Qing, G.; Duan, X.; Jiang, Y. *Yan Ke Xue Bao* **2005**, *21*, 163–168.
73. Aneja, R.; Hake, P. W.; Burroughs, T. J.; Denenberg, A. G.; Wong, H. R.; Zingarelli, B. *Mol. Med.* **2004**, *10*, 55–62.
74. Kang, K. S.; Wen, Y.; Yamabe, N.; Fukui, M.; Bishop, S. C.; Zhu, B. T. *PLoS One* **2010**, *5*, e11951.
75. Luo, T.; Wang, J.; Yin, Y.; Hua, H.; Jing, J.; Sun, X.; Li, M.; Zhang, Y.; Jiang, Y. *Breast Cancer Res.* **2010**, *12*, R8.
76. Harper, C. E.; Patel, B. B.; Wang, J.; Eltoum, I. A.; Lamartiniere, C. A. *Prostate* **2007**, *67*, 1576–1589.
77. Bae, H. B.; Li, M.; Kim, J. P.; Kim, S. J.; Jeong, C. W.; Lee, H. G.; Kim, W. M.; Kim, H. S.; Kwak, S. H. *Inflammation* **2010**, *33*, 82–91.
78. Goodin, M. G.; Rosengren, R. J. *Toxicol. Sci.* **2003**, *76*, 262–270.
79. Kalender, Y.; Yel, M.; Kalender, S. *Toxicology* **2005**, *209*, 39–45.
80. Ates, O.; Cayli, S.; Altinoz, E.; Gurses, I.; Yucel, N.; Sener, M.; Kocak, A.; Yologlu, S. *Mol. Cell. Biochem.* **2007**, *294*, 137–144.
81. Wang, Q.; Xu, J.; Rottinghaus, G. E.; Simonyi, A.; Lubahn, D.; Sun, G. Y.; Sun, A. Y. *Brain Res.* **2002**, *958*, 439–447.
82. Della-Morte, D.; Dave, K. R.; DeFazio, R. A.; Bao, Y. C.; Raval, A. P.; Perez-Pinzon, M. A. *Neuroscience* **2009**, *159*, 993–1002.
83. Jin, F.; Wu, Q.; Lu, Y. F.; Gong, Q. H.; Shi, J. S. *Eur. J. Pharmacol.* **2008**, *600*, 78–82.
84. Kumar, A.; Naidu, P. S.; Seghal, N.; Padi, S. S. *Pharmacology* **2007**, *79*, 17–26.
85. Kumar, P.; Padi, S. S.; Naidu, P. S.; Kumar, A. *Behav. Pharmacol.* **2006**, *17*, 485–492.
86. Wang, J.; Eltoum, I. E.; Lamartiniere, C. A. *J. Carcinogen.* **2007**, *6*, 3.
87. Vidlar, A.; Vostalova, J.; Ulrichova, J.; Student, V.; Krajicek, M.; Vrbkova, J.; Simanek, V. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Rep.* **2010**, *154*, 239–244.
88. Raina, K.; Blouin, M. J.; Singh, R. P.; Majeed, N.; Deep, G.; Varghese, L.; Glode, L. M.; Greenberg, N. M.; Hwang, D.; Cohen, P.; Pollak, M. N.; Agarwal, R. *Cancer Res.* **2007**, *67*, 11083–11091.
89. Galhardi, F.; Mesquita, K.; Monserrat, J. M.; Barros, D. M. *Food Chem. Toxicol.* **2009**, *47*, 2655–2660.
90. Ataie, A.; Sabetkasaei, M.; Haghparast, A.; Moghaddam, A. H.; Ataee, R.; Moghaddam, S. N. *J. Med. Food* **2010**, *13*, 821–826.
91. Ataie, A.; Sabetkasaei, M.; Haghparast, A.; Moghaddam, A. H.; Kazeminejad, B. *Pharmacol. Biochem. Behav.* **2010**, *96*, 378–385.
92. Kumar, P.; Padi, S. S.; Naidu, P. S.; Kumar, A. *Methods Find. Exp. Clin. Pharmacol.* **2007**, *29*, 19–25.
93. Gimeno, E.; de la Torre-Carbot, K.; Lamuela-Raventos, R. M.; Castellote, A. I.; Fito, M.; de la Torre, R.; Covas, M. I.; Lopez-Sabater, M. C. *Br. J. Nutr.* **2007**, *98*, 1243–1250.
94. Covas, M. I.; Nyyssonen, K.; Poulsen, H. E.; Kaikkonen, J.; Zunft, H. J.; Kiesewetter, H.; Gaddi, A.; de la Torre, R.; Mursu, J.; Baumler, H.; Nascetti, S.; Salonen, J. T.; Fito, M.; Virtanen, J.; Marrugat, J. *Ann. Intern. Med.* **2006**, *145*, 333–341.

95. Covas, M. I.; de la Torre, K.; Farre-Albaladejo, M.; Kaikkonen, J.; Fito, M.; Lopez-Sabater, C.; Pujadas-Bastardes, M. A.; Joglar, J.; Weinbrenner, T.; Lamuela-Raventos, R. M.; de la Torre, R. *Free Radical Biol. Med.* **2006**, *40*, 608–616.
96. Ruano, J.; Lopez-Miranda, J.; Fuentes, F.; Moreno, J. A.; Bellido, C.; Perez-Martinez, P.; Lozano, A.; Gomez, P.; Jimenez, Y.; Perez Jimenez, F. *J. Am. Coll. Cardiol.* **2005**, *46*, 1864–1868.
97. Fito, M.; Cladellas, M.; de la Torre, R.; Marti, J.; Alcantara, M.; Pujadas-Bastardes, M.; Marrugat, J.; Bruguera, J.; Lopez-Sabater, M. C.; Vila, J.; Covas, M. I. *Atherosclerosis* **2005**, *181*, 149–158.
98. Fito, M.; Cladellas, M.; de la Torre, R.; Marti, J.; Munoz, D.; Schroder, H.; Alcantara, M.; Pujadas-Bastardes, M.; Marrugat, J.; Lopez-Sabater, M. C.; Bruguera, J.; Covas, M. I. *Eur. J. Clin. Nutr.* **2008**, *62*, 570–574.
99. Leger, C. L.; Carbonneau, M. A.; Michel, F.; Mas, E.; Monnier, L.; Cristol, J. P.; Descomps, B. *Eur. J. Clin. Nutr.* **2005**, *59*, 727–730.
100. Machowetz, A.; Poulsen, H. E.; Gruendel, S.; Weimann, A.; Fito, M.; Marrugat, J.; de la Torre, R.; Salonen, J. T.; Nyyssonen, K.; Mursu, J.; Nascetti, S.; Gaddi, A.; Kiesewetter, H.; Baumler, H.; Selmi, H.; Kaikkonen, J.; Zunft, H. J.; Covas, M. I.; Koebnick, C. *FASEB J.* **2007**, *21*, 45–52.
101. Erba, D.; Riso, P.; Bordoni, A.; Foti, P.; Biagi, P. L.; Testolin, G. *J. Nutr. Biochem.* **2005**, *16*, 144–149.
102. Emara, A. M.; El-Bahrawy, H. *J. Immunotoxicol.* **2008**, *5*, 69–80.
103. Panza, V. S.; Wazlawik, E.; Ricardo Schutz, G.; Comin, L.; Hecht, K. C.; da Silva, E. L. *Nutrition* **2008**, *24*, 433–442.
104. Raederstorff, D. *Int. J. Vitam. Nutr. Res.* **2009**, *79*, 152–165.
105. Zhang, F.; Liu, J.; Shi, J. S. *Eur. J. Pharmacol.* **2010**, *636*, 1–7.
106. Kuriyama, S.; Shimazu, T.; Ohmori, K.; Kikuchi, N.; Nakaya, N.; Nishino, Y.; Tsubono, Y.; Tsuji, I. *J. Am. Med. Assoc.* **2006**, *296*, 1255–1265.
107. Frei, B.; Higdon, J. V. *J. Nutr.* **2003**, *133*, 3275S–3284S.
108. Dragsted, L. O. *Int. J. Vitam. Nutr. Res.* **2003**, *73*, 112–119.
109. Amorini, A. M.; Lazzarino, G.; Galvano, F.; Fazzina, G.; Tavazzi, B.; Galvano, G. *Free Radical Res.* **2003**, *37*, 453–460.
110. Gimeno, E.; de la Torre-Carbot, K.; Lamuela-Raventos, R. M.; Castellote, A. I.; Fito, M.; de la Torre, R.; Covas, M. I.; Lopez-Sabater, M. C. *Br. J. Nutr.* **2007**, *98*, 1243–1250.
111. Fito, M.; Cladellas, M.; de la Torre, R.; Marti, J.; Munoz, D.; Schroder, H.; Alcantara, M.; Pujadas-Bastardes, M.; Marrugat, J.; Lopez-Sabater, M. C.; Bruguera, J.; Covas, M. I. *Eur. J. Clin. Nutr.* **2008**, *62*, 570–574.
112. Williamson, G.; Manach, C. *Am. J. Clin. Nutr.* **2005**, *81*, 243S–255S.
113. Bhattacharya, U.; Halder, B.; Mukhopadhyay, S.; Giri, A. K. *Cancer Sci.* **2009**, *100*, 1971–1978.
114. Bobe, G.; Albert, P. S.; Sansbury, L. B.; Lanza, E.; Schatzkin, A.; Colburn, N. H.; Cross, A. J. *Cancer Prev. Res.* **2010**, *3*, 764–775.
115. Granado-Serrano, A. B.; Martin, M. A.; Bravo, L.; Goya, L.; Ramos, S. *Nutr. Cancer* **2010**, *62*, 390–401.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

116. Patel, R.; Krishnan, R.; Ramchandani, A.; Maru, G. *Cell Prolif.* **2008**, *41*, 532–553.
117. Prasad, S.; Kaur, J.; Roy, P.; Kalra, N.; Shukla, Y. *Life Sci.* **2007**, *81*, 1323–1331.
118. Coates, E. M.; Popa, G.; Gill, C. I.; McCann, M. J.; McDougall, G. J.; Stewart, D.; Rowland, I. *J. Carcinog.* **2007**, *6*, 4.
119. Vignes, M.; Maurice, T.; Lante, F.; Nedjar, M.; Thethi, K.; Guiramand, J.; Recasens, M. *Brain. Res.* **2006**, *1110*, 102–115.
120. Actis-Goretta, L.; Mackenzie, G. G.; Oteiza, P. I.; Fraga, C. G. *Ann. NY Acad. Sci.* **2002**, *957*, 279–283.
121. Abe, S.; Tanaka, Y.; Fujise, N.; Nakamura, T.; Masunaga, H.; Nagasawa, T.; Yagi, M. *J. Parenter. Enteral. Nutr.* **2007**, *31*, 181–187.
122. Basu, A.; Penugonda, K. *Nutr. Rev.* **2009**, *67*, 49–56.
123. Chang, W. H.; Huang, Y. F.; Yeh, T. S.; Hu, S. P.; Liu, J. F. *J. Appl. Physiol.* **2010**, *109*, 1710–1715.
124. Cheng, T. O. *Int. J. Cardiol.* **2006**, *108*, 301–308.
125. Hertog, M. G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. *Arch. Intern. Med.* **1995**, *155*, 381–386.
126. Dore, S. *Neurosignals* **2005**, *14*, 61–70.
127. Granados-Principal, S.; Quiles, J. L.; Ramirez-Tortosa, C. L.; Sanchez-Rovira, P.; Ramirez-Tortosa, M. C. *Food Chem. Toxicol.* **2010**, *48*, 1425–1438.
128. Holt, R. R.; Actis-Goretta, L.; Momma, T. Y.; Keen, C. L. *J. Cardiovasc. Pharmacol.* **2006**, *47*, S187–196.
129. Presti, R. L.; Carollo, C.; Caimi, G. *Nutrition* **2007**, *23*, 598–602.
130. Scalbert, A.; Manach, C.; Morand, C.; Remesy, C.; Jimenez, L. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306.
131. Huang, P. H.; Chen, Y. H.; Tsai, H. Y.; Chen, J. S.; Wu, T. C.; Lin, F. Y.; Sata, M.; Chen, J. W.; Lin, S. J. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 869–877.
132. Salvini, S.; Sera, F.; Caruso, D.; Giovannelli, L.; Visioli, F.; Saieva, C.; Masala, G.; Ceroti, M.; Giovacchini, V.; Pitozzi, V.; Galli, C.; Romani, A.; Mulinacci, N.; Bortolomeazzi, R.; Dolara, P.; Palli, D. *Br. J. Nutr.* **2006**, *95*, 742–751.
133. Sarr, M.; Chataigneau, M.; Martins, S.; Schott, C.; El Bedoui, J.; Oak, M. H.; Muller, B.; Chataigneau, T.; Schini-Kerth, V. B. *Cardiovasc. Res.* **2006**, *71*, 794–802.
134. Kuriyama, S.; Hozawa, A.; Ohmori, K.; Shimazu, T.; Matsui, T.; Ebihara, S.; Awata, S.; Nagatomi, R.; Arai, H.; Tsuji, I. *Am. J. Clin. Nutr.* **2006**, *83*, 355–361.
135. Sato, Y.; Nakatsuka, H.; Watanabe, T.; Hisamichi, S.; Shimizu, H.; Fujisaku, S.; Ichinowatari, Y.; Ida, Y.; Suda, S.; Kato, K. *Tohoku J. Exp. Med.* **1989**, *157*, 337–343.
136. Keli, S. O.; Hertog, M. G.; Feskens, E. J.; Kromhout, D. *Arch. Intern. Med.* **1996**, *156*, 637–642.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

137. Avramovich-Tirosh, Y.; Reznichenko, L.; Mit, T.; Zheng, H.; Fridkin, M.; Weinreb, O.; Mandel, S.; Youdim, M. B. *Curr. Alzheimer Res.* **2007**, *4*, 403–411.
138. de Souza-Pinto, N. C.; Wilson, D. M., 3rd; Stevnsner, T. V.; Bohr, V. A. *DNA Repair* **2008**, *7*, 1098–1109.
139. Lee, J. K.; Kwak, H. J.; Piao, M. S.; Jang, J. W.; Kim, S. H.; Kim, H. S. *Acta Neurochir.* **2011**, *153*, 1321–1329.
140. Weinreb, O.; Amit, T.; Mandel, S.; Youdim, M. B. *Genes Nutr.* **2009**, *4*, 283–296.
141. Mandel, S.; Maor, G.; Youdim, M. B. *J. Mol. Neurosci.* **2004**, *24*, 401–416.
142. Ikizler, M.; Erkasap, N.; Dernek, S.; Kural, T.; Kaygisiz, Z. *Anadolu Kardiyol. Derg.* **2007**, *7*, 404–410.
143. Perez-Vizcaino, F.; Duarte, J.; Andriantsitohaina, R. *Free Radical Res.* **2006**, *40*, 1054–1065.
144. Levites, Y.; Weinreb, O.; Maor, G.; Youdim, M. B.; Mandel, S. *J. Neurochem.* **2001**, *78*, 1073–1082.
145. Amazzal, L.; Lapotre, A.; Quignon, F.; Bagrel, D. *Neurosci. Lett.* **2007**, *418*, 159–164.
146. Dani, C.; Oliboni, L. S.; Vanderlinde, R.; Pra, D.; Dias, J. F.; Yoneama, M. L.; Bonatto, D.; Salvador, M.; Henriques, J. A. *J. Med. Food* **2009**, *12*, 188–192.
147. Mandel, S.; Weinreb, O.; Amit, T.; Youdim, M. B. *J. Neurochem.* **2004**, *88*, 1555–1569.
148. Seet, R. C.; Lee, C. Y.; Lim, E. C.; Tan, J. J.; Quek, A. M.; Chong, W. L.; Looi, W. F.; Huang, S. H.; Wang, H.; Chan, Y. H.; Halliwell, B. *Free Radical Biol. Med.* **2010**, *48*, 560–566.
149. Hu, G.; Bidel, S.; Jousilahti, P.; Antikainen, R.; Tuomilehto, J. *Mov. Disord.* **2007**, *22*, 2242–2248.
150. Karuppagounder, S. S.; Pinto, J. T.; Xu, H.; Chen, H. L.; Beal, M. F.; Gibson, G. E. *Neurochem. Int.* **2009**, *54*, 111–118.
151. Rovensky, J.; Stancikova, M.; Rovenska, E.; Stvrtina, S.; Stvrtinova, V.; Svik, K. *Ann. NY Acad. Sci.* **2009**, *1173*, 798–804.
152. Asghar, Z.; Masood, Z. *Pak. J. Pharm. Sci.* **2008**, *21*, 249–254.
153. Duong, T. T.; Antao, S.; Ellis, N. A.; Myers, S. J.; Witting, P. K. *Brain Res.* **2008**, *1219*, 8–18.
154. Mu, Y. L.; Xie, Y. Y.; Zhou, L.; Zhong, Y.; Liu, L.; Bai, H.; Wang, Y. S.; Zhang, X. M. *Chem. Biodiversity* **2009**, *6*, 1170–1177.
155. Matsuo, M. *J. Nutr. Sci. Vitaminol.* **2004**, *50*, 426–430.
156. Perron, N. R.; Hodges, J. N.; Jenkins, M.; Brumaghim, J. L. *Inorg. Chem.* **2008**, *47*, 6153–6161.
157. Pokrovsky, A. V.; Saveljev, V. S.; Kirienko, A. I.; Bogachev, V. Y.; Zolotukhin, I. A.; Sapelkin, S. V.; Shvalb, P. G.; Zhukov, B. N.; Vozlubleny, S. I.; Sabelnikov, V. V.; Voskanian, Y. E.; Katelnitsky, II; Burleva, E. P.; Tolstikhin, V. Y. *Angiol. Sosud. Khir.* **2007**, *13*, 47–55.
158. Rayner, B. S.; Duong, T. T.; Myers, S. J.; Witting, P. K. *J. Neurochem.* **2006**, *97*, 211–221.
159. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

160. Mu, Y.; Xie, Y.; Wang, F.; Zhong, Y.; Li, Z.; Hu, Z.; Wang, H.; Zhang, H. *J. Food Drug Anal.* **2009**, *17*, 11–16.
161. Bigelman, K. A.; Fan, E. H.; Chapman, D. P.; Freese, E. C.; Trilk, J. L.; Cureton, K. J. *Mil. Med.* **2010**, *175*, 791–798.
162. Davis, J. M.; Carlstedt, C. J.; Chen, S.; Carmichael, M. D.; Murphy, E. A. *Int. J. Sport Nutr. Exercise Metab.* **2010**, *20*, 56–62.
163. Ferruzzi, M. G.; Lobo, J. K.; Janle, E. M.; Cooper, B.; Simon, J. E.; Wu, Q. L.; Welch, C.; Ho, L.; Weaver, C.; Pasinetti, G. M. *J. Alzheimers Dis.* **2009**, *18*, 113–124.
164. Graefe, E. U.; Wittig, J.; Mueller, S.; Riethling, A. K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. *J. Clin. Pharmacol.* **2001**, *41*, 492–499.
165. Cerda, B.; Soto, C.; Albaladejo, M. D.; Martinez, P.; Sanchez-Gascon, F.; Tomas-Barberan, F.; Espin, J. C. *Eur. J. Clin. Nutr.* **2006**, *60*, 245–253.
166. Ajmo, J. M.; Liang, X.; Rogers, C. Q.; Pennock, B.; You, M. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2008**, *295*, G833–842.
167. Bobe, G.; Weinstein, S. J.; Albanes, D.; Hirvonen, T.; Ashby, J.; Taylor, P. R.; Virtamo, J.; Stolzenberg-Solomon, R. Z. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17*, 553–562.
168. Roy, P.; George, J.; Srivastava, S.; Tyagi, S.; Shukla, Y. *Invest. New Drugs* **2009**.
169. Visavadiya, N. P.; Soni, B.; Dalwadi, N. *Food Chem. Toxicol.* **2009**, *47*, 2507–2515.
170. Wang, L.; Tu, Y. C.; Lian, T. W.; Hung, J. T.; Yen, J. H.; Wu, M. J. *J. Agric. Food Chem.* **2006**, *54*, 9798–9804.
171. Christie, C. *J. Cardiovasc. Nurs.* **2010**, *25*, 233–237.
172. Levites, Y.; Amit, T.; Youdim, M. B.; Mandel, S. *J. Biol. Chem.* **2002**, *277*, 30574–30580.
173. Martin, L. D.; Krunkosky, T. M.; Dye, J. A.; Fischer, B. M.; Jiang, N. F.; Rochelle, L. G.; Akley, N. J.; Dreher, K. L.; Adler, K. B. *Environ. Health Perspect.* **1997**, *105*, 1301–1307.
174. Gao, Y. *Pflugers Arch.* **2010**, *459*, 829–839.
175. Lambert, A. J.; Brand, M. D. *Methods Mol. Biol.* **2009**, *554*, 165–181.
176. Gao, X.; Campian, J. L.; Qian, M.; Sun, X. F.; Eaton, J. W. *J. Biol. Chem.* **2009**, *284*, 4767–4775.
177. Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T. *Curr. Neuropharmacol.* **2009**, *7*, 65–74.
178. Huang, H.; Manton, K. G. *Front. Biosci.* **2004**, *9*, 1100–1117.
179. Imlay, J. A. *Annu. Rev. Biochem.* **2008**, *77*, 755–776.
180. Ballarin, L.; Cima, F.; Floreani, M.; Sabbadin, A. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* **2002**, *133*, 411–418.
181. Christophe, M.; Nicolas, S. *Curr. Pharm. Des.* **2006**, *12*, 739–757.
182. Liang, Q.; Dedon, P. C. *Chem. Res. Toxicol.* **2001**, *14*, 416–422.
183. Midorikawa, K.; Murata, M.; Kawanishi, S. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 1073–1077.
184. Turro, N. J. *Chem. Biol.* **2002**, *9*, 399–401.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

185. Filipcik, P.; Cente, M.; Ferencik, M.; Hulin, I.; Novak, M. *Bratisl. Lek. Listy* **2006**, *107*, 384–394.
186. Labinskyy, N.; Csiszar, A.; Veress, G.; Stef, G.; Pacher, P.; Oroszi, G.; Wu, J.; Ungvari, Z. *Curr. Med. Chem.* **2006**, *13*, 989–996.
187. Halliwell, B.; Aruoma, O. I. *FEBS Lett.* **1991**, *281*, 9–19.
188. Beckman, K. B.; Ames, B. N. *J. Biol. Chem.* **1997**, *272*, 19633–19636.
189. Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C. J.; Telser, J. *Mol. Cell. Biochem.* **2004**, *266*, 37–56.
190. Shen, J.; Deininger, P.; Hunt, J. D.; Zhao, H. *Cancer* **2007**, *109*, 574–580.
191. Murtas, D.; Piras, F.; Minerba, L.; Ugalde, J.; Floris, C.; Maxia, C.; Demurtas, P.; Perra, M. T.; Sirigu, P. *Oncol. Rep.* **2010**, *23*, 329–335.
192. Li, Y.; Cao, Z.; Zhu, H. *Pharmacol. Res.* **2006**, *53*, 6–15.
193. Robb, E. L.; Page, M. M.; Wiens, B. E.; Stuart, J. A. *Biochem. Biophys. Res. Commun.* **2008**, *367*, 406–412.
194. Imlay, J. A. *Adv. Microb. Physiol.* **2002**, *46*, 111–153.
195. Halliwell, B. *J. Neurochem.* **2006**, *97*, 1634–1658.
196. Yasui, H.; Hayashi, S.; Sakurai, H. *Drug Metab. Pharmacokinet.* **2005**, *20*, 1–13.
197. Li, C.; Wright, M. M.; Jackson, R. M. *Exp. Lung Res.* **2002**, *28*, 373–389.
198. Kwon, B. G.; Lee, J. H. *Anal. Chem.* **2004**, *76*, 6359–6364.
199. Tadolini, B.; Cabrini, L. *Mol. Cell. Biochem.* **1990**, *94*, 97–104.
200. Kim, Y. G.; Park, H. Y. *Phytother. Res.* **2004**, *18*, 900–905.
201. Saewong, T.; Ounjaijean, S.; Mundee, Y.; Pattanapanyasat, K.; Fucharoen, S.; Porter, J. B.; Srichairatanakool, S. *Med. Chem.* **2010**, *6*, 57–64.
202. Jomova, K.; Valko, M. *Toxicology* **2011**, *283*, 65–87.
203. Valko, M.; Rhodes, C. J.; Moncol, J.; Izakovic, M.; Mazur, M. *Chem. Biol. Interact.* **2006**, *160*, 1–40.
204. Huang, J.; Jones, D.; Luo, B.; Sanderson, M.; Soto, J.; Abel, E. D.; Cooksey, R. C.; McClain, D. A. *Diabetes* **2011**, *60*, 80–87.
205. Murphy, C. J.; Oudit, G. Y. *J. Card. Failure* **2010**, *16*, 888–900.
206. Park, J. H.; Park, E. *Mutat. Res.* **2011**, *718*, 56–61.
207. Daniel, K. G.; Harbach, R. H.; Guida, W. C.; Dou, Q. P. *Front. Biosci.* **2004**, *9*, 2652–2662.
208. Fahlman, B. M.; Krol, E. S. *J. Agric. Food Chem.* **2009**, *57*, 5301–5305.
209. Aquilano, K.; Baldelli, S.; Rotilio, G.; Ciriolo, M. R. *Neurochem. Res.* **2008**, *33*, 2416–2426.
210. Kim, Y. H.; Kim, Y. S.; Kang, S. S.; Cho, G. J.; Choi, W. S. *Diabetes* **2010**, *59*, 1825–1835.
211. Fraga, C. G. *IUBMB Life* **2007**, *59*, 308–315.
212. Es-Safi, N. E.; Ghidouche, S.; Ducrot, P. H. *Molecules* **2007**, *12*, 2228–2258.
213. Koren, E.; Kohen, R.; Ginsburg, I. *Exp. Biol. Med.* **2010**, *235*, 689–699.
214. Sang, S.; Hou, Z.; Lambert, J. D.; Yang, C. S. *Antioxid. Redox Signal.* **2005**, *7*, 1704–1714.
215. Iannelli, P.; Zarrilli, V.; Varricchio, E.; Tramontano, D.; Mancini, F. P. *Nutr. Metab. Cardiovasc. Dis.* **2007**, *17*, 247–256.

216. Smirnova, G. V.; Samoylova, Z. Y.; Muzyka, N. G.; Oktyabrsky, O. N. *Free Radical Biol Med.* **2009**, *46*, 759–768.
217. Vivancos, M.; Moreno, J. J. *Br. J. Nutr.* **2008**, *99*, 1199–1207.
218. Kidd, P. M. *Altern. Med. Rev.* **2009**, *14*, 226–246.
219. Terzuoli, E.; Donnini, S.; Giachetti, A.; Iniguez, M. A.; Fresno, M.; Melillo, G.; Ziche, M. *Clin. Cancer Res.* **2010**, *16*, 4207–4216.
220. Shanu, A.; Parry, S. N.; Wood, S.; Rodas, E.; Witting, P. K. *Free Radical Res.* **2010**, *44*, 843–853.
221. Moon, J. K.; Shibamoto, T. *J. Agric. Food Chem.* **2009**, *57*, 1655–1666.
222. Okawa, M.; Kinjo, J.; Nohara, T.; Ono, M. *Biol. Pharm. Bull.* **2001**, *24*, 1202–1205.
223. Weisel, T.; Baum, M.; Eisenbrand, G.; Dietrich, H.; Will, F.; Stockis, J. P.; Kulling, S.; Rufer, C.; Johannes, C.; Janzowski, C. *Biotechnol. J.* **2006**, *1*, 388–397.
224. Lucarini, M.; Pedulli, G. F.; Guerra, M. *Chemistry* **2004**, *10*, 933–939.
225. Kaur, I. P.; Geetha, T. *Mini Rev. Med. Chem.* **2006**, *6*, 305–312.
226. Wilms, L. C.; Hollman, P. C.; Boots, A. W.; Kleinjans, J. C. *Mutat. Res.* **2005**, *582*, 155–162.
227. Glei, M.; Pool-Zobel, B. L. *Toxicol. in Vitro* **2006**, *20*, 295–300.
228. Rahman, M. M.; Ichiyanagi, T.; Komiyama, T.; Hatano, Y.; Konishi, T. *Free Radical Res.* **2006**, *40*, 993–1002.
229. Dietrich-Muszalska, A.; Olas, B. *World J. Biol. Psychiatry* **2010**, *11*, 276–281.
230. El-Agamy, D. S. *Arch. Toxicol.* **2010**, *84*, 389–396.
231. Lodi, F.; Jimenez, R.; Moreno, L.; Kroon, P. A.; Needs, P. W.; Hughes, D. A.; Santos-Buelga, C.; Gonzalez-Paramas, A.; Cogolludo, A.; Lopez-Sepulveda, R.; Duarte, J.; Perez-Vizcaino, F. *Atherosclerosis* **2009**, *204*, 34–39.
232. Faine, L. A.; Rodrigues, H. G.; Galhardi, C. M.; Ebaid, G. M.; Diniz, Y. S.; Padovani, C. R.; Novelli, E. L. *Can. J. Physiol. Pharmacol.* **2006**, *84*, 239–245.
233. Celik, H.; Arinc, E. *J. Pharm. Pharm. Sci.* **2010**, *13*, 231–241.
234. Koksal, E.; Gulcin, I.; Beyza, S.; Sarikaya, O.; Bursal, E. J. *Enzyme Inhib. Med. Chem.* **2009**, *24*, 395–405.
235. Mladenka, P.; Zatloukalova, L.; Filipsky, T.; Hrdina, R. *Free Radical Biol. Med.* **2010**, *49*, 963–975.
236. Romero, M.; Jimenez, R.; Sanchez, M.; Lopez-Sepulveda, R.; Zarzuelo, M. J.; O'Valle, F.; Zarzuelo, A.; Perez-Vizcaino, F.; Duarte, J. *Atherosclerosis* **2009**, *202*, 58–67.
237. Alia, M.; Mateos, R.; Ramos, S.; Lecumberri, E.; Bravo, L.; Goya, L. *Eur. J. Nutr.* **2006**, *45*, 19–28.
238. Granado-Serrano, A. B.; Martin, M. A.; Bravo, L.; Goya, L.; Ramos, S. *J. Nutr.* **2006**, *136*, 2715–2721.
239. Kim, M.; Murakami, A.; Kawabata, K.; Ohigashi, H. *Carcinogenesis* **2005**, *26*, 1553–1562.
240. Xu, J. W.; Ikeda, K.; Yamori, Y. *Hypertension* **2004**, *44*, 217–222.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

241. Visavadiya, N. P.; Narasimhacharya, A. V. *Food Chem. Toxicol.* **2008**, *46*, 1889–1895.
242. Qian, L. B.; Wang, H. P.; Chen, Y.; Chen, F. X.; Ma, Y. Y.; Bruce, I. C.; Xia, Q. *Pharmacol. Res.* **2010**, *61*, 281–287.
243. Sil, H.; Sen, T.; Moulik, S.; Chatterjee, A. *J. Environ. Pathol. Toxicol. Oncol.* **2010**, *29*, 55–68.
244. Fernandez, M. T.; Mira, M. L.; Florencio, M. H.; Jennings, K. R. *J. Inorg. Biochem.* **2002**, *92*, 105–111.
245. Perron, N. R.; Wang, H. C.; Deguire, S. N.; Jenkins, M.; Lawson, M.; Brumaghim, J. L. *Dalton Trans.* **2010**, *39*, 9982–9987.
246. Sestili, P.; Guidarelli, A.; Dacha, M.; Cantoni, O. *Free Radical Biol. Med.* **1998**, *25*, 196–200.
247. Mira, L.; Fernandez, M. T.; Santos, M.; Rocha, R.; Florencio, M. H.; Jennings, K. R. *Free Radical Res.* **2002**, *36*, 1199–1208.
248. van Acker, F. A.; Hulshof, J. W.; Haenen, G. R.; Menge, W. M.; van der Vijgh, W. J.; Bast, A. *Free Radical Biol. Med.* **2001**, *31*, 31–37.
249. Sugihara, N.; Arakawa, T.; Ohnishi, M.; Furuno, K. *Free Radical Biol. Med.* **1999**, *27*, 1313–1323.
250. Kazazic, S. P.; Butkovic, V.; Srzic, D.; Klasinc, L. *J. Agric. Food Chem.* **2006**, *54*, 8391–8396.
251. Sahu, S. C.; Ruggles, D. I.; O'Donnell, M. W. *Food Chem. Toxicol.* **2006**, *44*, 1751–1757.
252. Laughton, M. J.; Halliwell, B.; Evans, P. J.; Hoult, J. R. *Biochem. Pharmacol.* **1989**, *38*, 2859–2865.
253. Behari, M.; Pardasani, V. *Parkinsonism Relat. Disord.* **2010**, *16*, 639–644.
254. Halliwell, B. *J. Neurochem.* **1992**, *59*, 1609–1623.
255. Boveris, A. D.; Galleano, M.; Puntarulo, S. *Phytother. Res.* **2007**, *21*, 735–740.
256. Brown, K. E.; Meleah Mathahs, M.; Broadhurst, K. A.; Coleman, M. C.; Ridnour, L. A.; Schmidt, W. N.; Spitz, D. R. *Free Radical Biol. Med.* **2007**, *42*, 228–235.
257. Cronje, L.; Edmondson, N.; Eisenach, K. D.; Bornman, L. *FEMS Immunol. Med. Microbiol.* **2005**, *45*, 103–112.
258. Gharagozloo, M.; Khoshdel, Z.; Amirghofran, Z. *Eur. J. Pharmacol.* **2008**, *589*, 1–7.
259. Chen, T. S.; Liou, S. Y.; Chang, Y. L. *Am. J. Chin. Med.* **2008**, *36*, 1209–1217.
260. Bernardo, L. C.; de Oliveira, M. B.; da Silva, C. R.; Dantas, F. J.; de Matros, J. C.; Caldeira-de-Araujo, A.; Moura, R. S.; Bernardo-Filho, M. *Cell. Mol. Biol.* **2002**, *48*, 517–520.
261. Toledo, M. A.; Schneider, D. R.; Azzoni, A. R.; Favaro, M. T.; Pelloso, A. C.; Santos, C. A.; Saraiva, A. M.; Souza, A. P. *Protein Expr. Purif.* **2011**, *75*, 204–210.
262. Messner, K. R.; Imlay, J. A. *J. Biol. Chem.* **2002**, *277*, 42563–42571.
263. Kthiri, F.; Le, H. T.; Gautier, V.; Caldas, T.; Malki, A.; Landoulsi, A.; Bohn, C.; Bouloc, P.; Richarme, G. *J. Biol. Chem.* **2010**, *285*, 10328–10336.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

264. Malik, R.; Kashyap, A.; Bansal, K.; Sharma, P.; Rayasam, G. V.; Davis, J. A.; Bora, R. S.; Ray, A.; Saini, K. S. *Biochem. Biophys. Res. Commun.* **2010**, *391*, 739–743.

265. Mukai, N.; Masaki, K.; Fujii, T.; Kawamukai, M.; Iefuji, H. *J. Biosci. Bioeng.* **2010**, *109*, 564–569.

266. Schellhorn, H. E.; Hassan, H. M. *Can. J. Microbiol.* **1988**, *34*, 1171–1176.

267. McLean, S.; Bowman, L. A.; Sanguinetti, G.; Read, R. C.; Poole, R. K. *J. Biol. Chem.* **2010**, *285*, 20724–20731.

268. Oktyabrsky, O.; Vysochina, G.; Muzyka, N.; Samoilova, Z.; Kukushkina, T.; Smirnova, G. *J. Appl. Microbiol.* **2009**, *106*, 1175–1183.

269. Semchyshyn, H. M.; Lushchak, V. I. *Ukr. Biokhim. Zh.* **2004**, *76*, 31–42.

270. Storz, G.; Tartaglia, L. A.; Ames, B. N. *Antonie van Leeuwenhoek* **1990**, *58*, 157–161.

271. Smith, A. H.; Imlay, J. A.; Mackie, R. I. *Appl. Environ. Microbiol.* **2003**, *69*, 3406–3411.

272. Chary, P.; Dillon, D.; Schroeder, A. L.; Natvig, D. O. *Genetics* **1994**, *137*, 723–730.

273. Demple, B.; Hidalgo, E.; Ding, H. *Biochem. Soc. Symp.* **1999**, *64*, 119–128.

274. Molina-Heredia, F. P.; Houee-Levin, C.; Berthomieu, C.; Touati, D.; Tremey, E.; Favaudon, V.; Adam, V.; Niviere, V. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 14750–14755.

275. Kogoma, T.; Yura, T. *J. Bacteriol.* **1992**, *174*, 630–632.

276. Mapson, L. W.; Ingram, M. *Biochem. J.* **1951**, *48*, 551–559.

277. SciFinder search on April 28, 2011, using the terms "bacterial" and "*E. coli*", refined by article and reviews only (duplicate entries removed).

278. Dadi, P. K.; Ahmad, M.; Ahmad, Z. *Int. J. Biol. Macromol.* **2009**, *45*, 72–79.

279. Larrosa, M.; Gonzalez-Sarrias, A.; Yanez-Gascon, M. J.; Selma, M. V.; Azorin-Ortuno, M.; Toti, S.; Tomas-Barberan, F.; Dolara, P.; Espin, J. C. *J. Nutr. Biochem.* **2010**, *21*, 717–725.

280. Martin, A. R.; Villegas, I.; La Casa, C.; de la Lastra, C. A. *Biochem. Pharmacol.* **2004**, *67*, 1399–1410.

281. Ingham, S. C.; Algino, R. J.; Ingham, B. H.; Schell, R. F. *J. Food Prot.* **2010**, *73*, 1864–1874.

282. Tromp, S. O.; Rijgersberg, H.; Franz, E. *J. Food Prot.* **2010**, *73*, 1830–1840.

283. Viazis, S.; Akhtar, M.; Feirtag, J.; Diez-Gonzalez, F. *Food Microbiol.* **2010**, *28*, 149–157.

284. Nakao, M.; Takio, S.; Ono, K. *Phytochemistry* **1998**, *49*, 2379–2382.

285. Park, S. J.; Chung, H. Y.; Lee, J. H. *J. Appl. Microbiol.* **2010**, *108*, 1217–1225.

286. Park, S. J.; Chung, H. Y.; Lee, J. H. *J. Appl. Microbiol.* **2010**, *108*, 1217–1225.

287. Skandrani, I.; Limem, I.; Neffati, A.; Boubaker, J.; Ben Sghaier, M.; Bhouri, W.; Bouhlel, I.; Kilani, S.; Ghedira, K.; Chekir-Ghedira, L. *Food Chem. Toxicol.* **2010**, *48*, 710–715.

288. Eberhardt, M. V.; Kobira, K.; Keck, A. S.; Juvik, J. A.; Jeffery, E. H. *J. Agric. Food Chem.* **2005**, *53*, 7421–7431.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

289. Smirnova, G. V.; Vysochina, G. I.; Muzyka, N. G.; Samoilova, Z.; Kukushkina, T. A.; Oktiabr'skii, O. N. *Prikl. Biokhim. Mikrobiol.* **2009**, *45*, 705–709.
290. Tian, X. J.; Yang, X. W.; Yang, X.; Wang, K. *Int. J. Pharm.* **2009**, *367*, 58–64.
291. Bouaziz, M.; Lassoued, S.; Bouallagui, Z.; Smaoui, S.; Gargoubi, A.; Dhouib, A.; Sayadi, S. *Bioorg. Med. Chem.* **2008**, *16*, 9238–9246.
292. Urios, A.; Lopez-Gresa, M. P.; Gonzalez, M. C.; Primo, J.; Martinez, A.; Herrera, G.; Escudero, J. C.; O'Connor, J. E.; Blanco, M. *Free Radical Biol. Med.* **2003**, *35*, 1373–1381.
293. Makena, P. S.; Pierce, S. C.; Chung, K. T.; Sinclair, S. E. *Environ. Mol. Mutagen.* **2009**, *50*, 451–459.
294. Hickey, E. K.; Cianciotto, N. P. *Infect. Immun.* **1997**, *65*, 133–143.
295. Tardat, B.; Touati, D. *Mol. Microbiol.* **1991**, *5*, 455–465.
296. Ounjaijean, S.; Thephinlap, C.; Khansuwan, U.; Phisalapong, C.; Fucharoen, S.; Porter, J. B.; Srichairatanakool, S. *Med. Chem.* **2008**, *4*, 365–370.
297. Pekal, A.; Biesaga, M.; Pyrzynska, K. *Biometals* **2011**, *24*, 41–49.
298. Mandel, S.; Amit, T.; Reznichenko, L.; Weinreb, O.; Youdim, M. B. *Mol. Nutr. Food Res.* **2006**, *50*, 229–234.
299. Woodmansee, A. N.; Imlay, J. A. *J. Biol. Chem.* **2002**, *277*, 34055–34066.
300. Yoshino, M.; Haneda, M.; Naruse, M.; Murakami, K. *Mol. Genet. Metab.* **1999**, *68*, 468–472.
301. Pekal, A.; Biesaga, M.; Pyrzynska, K. *Biometals* **2010**.
302. Martinez, A.; Urios, A.; Blanco, M. *Mutat. Res.* **2000**, *467*, 41–53.
303. Grazula, M.; Budzisza, E. *Coord. Chem. Rev.* **2009**, *253*, 2588–2598.
304. Campbell, Z. T.; Baldwin, T. O. *J. Biol. Chem.* **2009**, *284*, 8322–8328.
305. Urios, A.; Largeron, M.; Fleury, M. B.; Blanco, M. *Free Radical Biol. Med.* **2006**, *40*, 791–800.
306. Mates, J. M.; Segura, J. A.; Alonso, F. J.; Marquez, J. *Arch. Toxicol.* **2008**, *82*, 273–299.
307. Nohl, H.; Gille, L.; Kozlov, A.; Staniek, K. *Redox Rep.* **2003**, *8*, 135–141.
308. Heise, K.; Puntarulo, S.; Portner, H. O.; Abele, D. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2003**, *134*, 79–90.
309. Akbari, M.; Visnes, T.; Krokan, H. E.; Otterlei, M. *DNA Repair* **2008**, *7*, 605–616.
310. Gao, J.; Zhao, N.; Knutson, M. D.; Enns, C. A. *J. Biol. Chem.* **2008**, *283*, 21462–21468.
311. Petrak, J.; Myslivcova, D.; Man, P.; Cmejla, R.; Cmejlova, J.; Vyoral, D. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *290*, G1059–1066.
312. Kirsch, R.; Sijtsema, H. P.; Tlali, M.; Marais, A. D.; Hall Pde, L. *Liver Int.* **2006**, *26*, 1258–1267.
313. Mizukami, S.; Ichimura, R.; Kemmochi, S.; Wang, L.; Taniai, E.; Mitsumori, K.; Shibutani, M. *Chem. Biol. Interact.* **2010**, *185*, 189–201.
314. Choi, E. H.; Chang, H. J.; Cho, J. Y.; Chun, H. S. *Food Chem. Toxicol.* **2007**, *45*, 1873–1881.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

315. Spormann, T. M.; Albert, F. W.; Rath, T.; Dietrich, H.; Will, F.; Stockis, J. P.; Eisenbrand, G.; Janzowski, C. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17*, 3372–3380.
316. Poli, G.; Leonarduzzi, G.; Biasi, F.; Chiarpotto, E. *Curr. Med. Chem.* **2004**, *11*, 1163–1182.
317. Benito, S.; Lopez, D.; Saiz, M. P.; Buxaderas, S.; Sanchez, J.; Puig-Parellada, P.; Mitjavila, M. T. *Br. J. Pharmacol.* **2002**, *135*, 910–916.
318. Martinez-Florez, S.; Gutierrez-Fernandez, B.; Sanchez-Campos, S.; Gonzalez-Gallego, J.; Tunon, M. J. *J. Nutr.* **2005**, *135*, 1359–1365.
319. Liang, Y. C.; Huang, Y. T.; Tsai, S. H.; Lin-Shiau, S. Y.; Chen, C. F.; Lin, J. K. *Carcinogenesis* **1999**, *20*, 1945–1952.
320. Shen, S. C.; Lee, W. R.; Lin, H. Y.; Huang, H. C.; Ko, C. H.; Yang, L. L.; Chen, Y. C. *Eur. J. Pharmacol.* **2002**, *446*, 187–194.
321. Kim, B. H.; Cho, S. M.; Reddy, A. M.; Kim, Y. S.; Min, K. R.; Kim, Y. *Biochem. Pharmacol.* **2005**, *69*, 1577–1583.
322. Garcia-Mediavilla, V.; Crespo, I.; Collado, P. S.; Esteller, A.; Sanchez-Campos, S.; Tunon, M. J.; Gonzalez-Gallego, J. *Eur. J. Pharmacol.* **2007**, *557*, 221–229.
323. Chen, C. Y.; Peng, W. H.; Tsai, K. D.; Hsu, S. L. *Life Sci.* **2007**, *81*, 1602–1614.
324. Mutoh, M.; Takahashi, M.; Fukuda, K.; Matsushima-Hibiya, Y.; Mutoh, H.; Sugimura, T.; Wakabayashi, K. *Carcinogenesis* **2000**, *21*, 959–963.
325. Hou, D. X.; Masuzaki, S.; Hashimoto, F.; Uto, T.; Tanigawa, S.; Fujii, M.; Sakata, Y. *Arch. Biochem. Biophys.* **2007**, *460*, 67–74.
326. Camacho-Barquero, L.; Villegas, I.; Sanchez-Calvo, J. M.; Talero, E.; Sanchez-Fidalgo, S.; Motilva, V.; Alarcon de la Lastra, C. *Int. Immunopharmacol.* **2007**, *7*, 333–342.
327. Hong, J.; Bose, M.; Ju, J.; Ryu, J. H.; Chen, X.; Sang, S.; Lee, M. J.; Yang, C. S. *Carcinogenesis* **2004**, *25*, 1671–1679.
328. Rao, C. V. *Adv. Exp. Med. Biol.* **2007**, *595*, 213–226.
329. Kim, J. S.; Kim, J. C.; Shim, S. H.; Lee, E. J.; Jin, W.; Bae, K.; Son, K. H.; Kim, H. P.; Kang, S. S.; Chang, H. W. *Arch. Pharm. Res.* **2006**, *29*, 617–623.
330. Lin, C. W.; Hou, W. C.; Shen, S. C.; Juan, S. H.; Ko, C. H.; Wang, L. M.; Chen, Y. C. *Carcinogenesis* **2008**, *29*, 1807–1815.
331. Chinnam, N.; Dadi, P. K.; Sabri, S. A.; Ahmad, M.; Kabir, M. A.; Ahmad, Z. *Int. J. Biol. Macromol.* **2010**, *46*, 478–486.
332. Nessa, F.; Ismail, Z.; Mohamed, N. *Pharm. Biol.* **2010**, *48*, 1405–1412.
333. Alvarez, P.; Alvarado, C.; Mathieu, F.; Jimenez, L.; De la Fuente, M. *Eur. J. Nutr.* **2006**, *45*, 428–438.
334. Jung, Y. H.; Heo, J.; Lee, Y. J.; Kwon, T. K.; Kim, Y. H. *Life Sci.* **2010**, *86*, 351–357.
335. Harasstani, O. A.; Moin, S.; Tham, C. L.; Liew, C. Y.; Ismail, N.; Rajajendram, R.; Harith, H. H.; Zakaria, Z. A.; Mohamad, A. S.; Sulaiman, M. R.; Israf, D. A. *Inflamm. Res.* **2010**, *59*, 711–721.
336. Wheeler, D. S.; Catravas, J. D.; Odoms, K.; Denenberg, A.; Malhotra, V.; Wong, H. R. *J. Nutr.* **2004**, *134*, 1039–1044.

337. Gupta, A.; Vij, G.; Sharma, S.; Tirkey, N.; Rishi, P.; Chopra, K. *Immunobiology* **2009**, *214*, 33–39.
338. Tribolo, S.; Lodi, F.; Connor, C.; Suri, S.; Wilson, V. G.; Taylor, M. A.; Needs, P. W.; Kroon, P. A.; Hughes, D. A. *Atherosclerosis* **2008**, *197*, 50–56.
339. Sakurai, T.; Kitadate, K.; Nishioka, H.; Fujii, H.; Kizaki, T.; Kondoh, Y.; Izawa, T.; Ishida, H.; Radak, Z.; Ohno, H. *J. Nutr. Biochem.* **2010**, *21*, 47–54.
340. Rajapakse, A. G.; Ming, X. F.; Carvas, J. M.; Yang, Z. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296*, H815–822.
341. Buffoli, B.; Pechanova, O.; Kojsova, S.; Andriantsitohaina, R.; Giugno, L.; Bianchi, R.; Rezzani, R. *J. Histochem. Cytochem.* **2005**, *53*, 1459–1468.
342. Zhong, Z.; Froh, M.; Lehnert, M.; Schoonhoven, R.; Yang, L.; Lind, H.; Lemasters, J. J.; Thurman, R. G. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2003**, *285*, G1004–1013.
343. Shiner, M.; Fuhrman, B.; Aviram, M. *Atherosclerosis* **2007**, *195*, 313–321.
344. Sharma, M.; Manoharlal, R.; Puri, N.; Prasad, R. *Biosci. Rep.* **2010**, *30*, 391–404.
345. Boadi, W. Y.; Iyere, P. A.; Adunyah, S. E. *J. Appl. Toxicol.* **2003**, *23*, 363–369.
346. Abbas, S.; Wink, M. *Planta Med.* **2009**, *75*, 216–221.
347. Waldmeier, F.; Bruin, G. J.; Glaenzel, U.; Hazell, K.; Sechaud, R.; Warrington, S.; Porter, J. B. *Drug Metab. Dispos.* **2010**, *38*, 808–816.
348. Katiyar, S. K.; Afaq, F.; Azizuddin, K.; Mukhtar, H. *Toxicol. Appl. Pharmacol.* **2001**, *176*, 110–117.
349. Feng, R.; Ni, H. M.; Wang, S. Y.; Tourkova, I. L.; Shurin, M. R.; Harada, H.; Yin, X. M. *J. Biol. Chem.* **2007**, *282*, 13468–13476.
350. Moon, T. C.; Kim, J. C.; Song, S. E.; Suh, S. J.; Seo, C. S.; Kim, Y. K.; Jin, M.; Yang, J. H.; Son, J. K.; Jahng, Y.; Kim, C. H.; Chang, H. W. *Int. Immunopharmacol.* **2008**, *8*, 1395–1400.
351. Hung, Y. C.; Wang, P. W.; Pan, T. L.; Bazylak, G.; Leu, Y. L. *J. Ethnopharmacol.* **2009**, *124*, 463–474.
352. Sanchez-Fidalgo, S.; Cardeno, A.; Villegas, I.; Talero, E.; de la Lastra, C. A. *Eur. J. Pharmacol.* **2010**, *633*, 78–84.
353. Chen, M.; Hu, D. N.; Pan, Z.; Lu, C. W.; Xue, C. Y.; Aass, I. *Exp. Eye Res.* **2010**, *90*, 437–443.
354. Godenschwege, T.; Forde, R.; Davis, C. P.; Paul, A.; Beckwith, K.; Duttaroy, A. *Genetics* **2009**, *183*, 175–184.
355. Ullah, M. F.; Shamim, U.; Hanif, S.; Azmi, A. S.; Hadi, S. M. *Mol. Nutr. Food Res.* **2009**, *53*, 1376–1385.
356. Kapiszewska, M.; Cierniak, A.; Elas, M.; Lankoff, A. *Toxicol. in Vitro* **2007**, *21*, 1020–1030.
357. Madeira, S. V.; Auger, C.; Anselm, E.; Chataigneau, M.; Chataigneau, T.; Soares de Moura, R.; Schini-Kerth, V. B. *J. Vasc. Res.* **2009**, *46*, 406–416.
358. D'Angelo, S.; Morana, A.; Salvatore, A.; Zappia, V.; Galletti, P. *J. Med. Food* **2009**, *12*, 1326–1333.
359. Granado-Serrano, A. B.; Martin, M. A.; Haegeman, G.; Goya, L.; Bravo, L.; Ramos, S. *Br. J. Nutr.* **2010**, *103*, 168–179.
360. Poudyal, H.; Campbell, F.; Brown, L. *J. Nutr.* **2010**, *140*, 946–953.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

361. Wang, P.; Aronson, W. J.; Huang, M.; Zhang, Y.; Lee, R. P.; Heber, D.; Henning, S. M. *Cancer Prev. Res.* **2010**, *3*, 985–993.
362. Apak, R.; Guclu, K.; Demirata, B.; Ozyurek, M.; Celik, S. E.; Bektasoglu, B.; Berker, K. I.; Ozyurt, D. *Molecules* **2007**, *12*, 1496–1547.
363. Lozano, C.; Julia, L.; Jimenez, A.; Tourino, S.; Centelles, J. J.; Cascante, M.; Torres, J. L. *FEBS J.* **2006**, *273*, 2475–2486.
364. Hirano, R.; Sasamoto, W.; Matsumoto, A.; Itakura, H.; Igarashi, O.; Kondo, K. *J. Nutr. Sci. Vitaminol.* **2001**, *47*, 357–362.
365. Galleano, M.; Verstraeten, S. V.; Oteiza, P. I.; Fraga, C. G. *Arch. Biochem. Biophys.* **2010**, *501*, 23–30.
366. Furuno, K.; Akasako, T.; Sugihara, N. *Biol. Pharm. Bull.* **2002**, *25*, 19–23.
367. Sorata, Y.; Takahama, U.; Kimura, M. *Biochim. Biophys. Acta* **1984**, *799*, 313–317.
368. Solomon, A.; Golubowicz, S.; Yablowicz, Z.; Bergman, M.; Grossman, S.; Altman, A.; Kerem, Z.; Flaishman, M. A. *J. Agric. Food Chem.* **2010**, *58*, 7158–7165.
369. Yokomizo, A.; Moriwaki, M. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1317–1324.
370. Murzakhmetova, M.; Moldakarimov, S.; Tancheva, L.; Abarova, S.; Serkedjieva, J. *Phytother. Res.* **2008**, *22*, 746–751.
371. Breborowicz, M.; Polubinska, A.; Tam, P.; Wu, G.; Breborowicz, A. *Eur. J. Clin. Invest.* **2003**, *33*, 1038–1044.
372. Owen, P. L.; Matainaho, T.; Sirois, M.; Johns, T. *J. Biochem. Mol. Toxicol.* **2007**, *21*, 231–242.
373. Psotova, J.; Chlopcikova, S.; Grambal, F.; Simanek, V.; Ulrichova, J. *Phytother. Res.* **2002**, *16*, S63–67.
374. Saller, R.; Meier, R.; Brignoli, R. *Drugs* **2001**, *61*, 2035–2063.
375. Schomburg, I.; Chang, A.; Ebeling, C.; Gremse, M.; Heldt, C.; Huhn, G.; Schomburg, D. *Nucleic Acids Res.* **2004**, *32*, D431–D433.
376. Griner, E. M.; Kazanietz, M. G. *Nat. Rev. Cancer* **2007**, *7*, 281–294.
377. Pignatelli, P.; Di Santo, S.; Buchetti, B.; Sanguigni, V.; Brunelli, A.; Violi, F. *FASEB J.* **2006**, *20*, 1082–1089.
378. Wilkinson, A. P.; Gee, J. M.; Dupont, M. S.; Needs, P. W.; Mellon, F. A.; Williamson, G.; Johnson, I. T. *Xenobiotica* **2003**, *33*, 255–264.
379. Asano, J.; Niisato, N.; Nakajima, K.; Miyazaki, H.; Yasuda, M.; Iwasaki, Y.; Hama, T.; Dejima, K.; Hisa, Y.; Marunaka, Y. *Am. J. Respir. Cell. Mol. Biol.* **2009**, *41*, 688–695.
380. Zheng, Y.; Toborek, M.; Hennig, B. *Metabolism* **2010**, *59*, 1528–1535.
381. Takada, Y.; Sethi, G.; Sung, B.; Aggarwal, B. B. *Mol. Pharmacol.* **2008**, *73*, 1549–1557.
382. Wu, C. C.; Hsu, M. C.; Hsieh, C. W.; Lin, J. B.; Lai, P. H.; Wung, B. S. *Life Sci.* **2006**, *78*, 2889–2897.
383. Dargelos, E.; Brule, C.; Stuelsatz, P.; Mouly, V.; Veschambre, P.; Cottin, P.; Poussard, S. *Exp. Cell. Res.* **2010**, *316*, 115–125.
384. Lorenz, M.; Hellige, N.; Rieder, P.; Kinkel, H. T.; Trimpert, C.; Staudt, A.; Felix, S. B.; Baumann, G.; Stangl, K.; Stangl, V. *Eur. J. Heart Failure* **2008**, *10*, 439–445.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

385. Nicholson, S. K.; Tucker, G. A.; Brameld, J. M. *Br. J. Nutr.* **2010**, *103*, 1398–1403.
386. Lorenz, M.; Wessler, S.; Follmann, E.; Michaelis, W.; Dusterhoft, T.; Baumann, G.; Stangl, K.; Stangl, V. *J. Biol. Chem.* **2004**, *279*, 6190–6195.
387. Sebai, H.; Sani, M.; Ghanem-Boughanmi, N.; Aouani, E. *Food Chem. Toxicol.* **2010**, *48*, 1543–1549.
388. Ahmad, Z.; Laughlin, T. F. *Curr. Med. Chem.* **2010**, *17*, 2822–2836.
389. Aucamp, J.; Gaspar, A.; Hara, Y.; Apostolides, Z. *Anticancer Res.* **1997**, *17*, 4381–4385.
390. Chander, V.; Chopra, K. *J. Vasc. Surg.* **2005**, *42*, 1198–1205.
391. Hanneken, A.; Lin, F. F.; Johnson, J.; Maher, P. *Invest. Ophthalmol. Vis. Sci.* **2006**, *47*, 3164–3177.
392. Sestili, P.; Diamantini, G.; Bedini, A.; Cerioni, L.; Tommasini, I.; Tarzia, G.; Cantoni, O. *Biochem. J.* **2002**, *364*, 121–128.
393. Sestili, P.; Martinelli, C.; Ricci, D.; Fraternale, D.; Bucchini, A.; Giamperi, L.; Curcio, R.; Piccoli, G.; Stocchi, V. *Pharmacol. Res.* **2007**, *56*, 18–26.
394. Lu, Z.; Nie, G.; Belton, P. S.; Tang, H.; Zhao, B. *Neurochem. Int.* **2006**, *48*, 263–274.
395. Boadi, W. Y.; Iyere, P. A.; Adunyah, S. E. *J. Appl. Toxicol.* **2005**, *25*, 82–88.
396. Cristina Megías, E. P.-C.; Torres-Fuentes, Cristina; Girï¿½n-Calle, Julio; Alaiz, Manuel; Juan, Rocio; Pastor, Julio; Vioque, Javier *Eur. Food Res. Technol.* **2009**, *230*, 353–359.
397. Srichairatanakool, S.; Ounjaijean, S.; Thephinlap, C.; Khansuwan, U.; Phisalpong, C.; Fucharoen, S. *Hemoglobin* **2006**, *30*, 311–327.
398. Thephinlap, C.; Ounjaijean, S.; Khansuwan, U.; Fucharoen, S.; Porter, J. B.; Srichairatanakool, S. *Med. Chem.* **2007**, *3*, 289–296.
399. Thephinlap, C.; Phisalaphong, C.; Fucharoen, S.; Porter, J. B.; Srichairatanakool, S. *Med. Chem.* **2009**, *5*, 474–482.
400. Quesada, I. M.; Bustos, M.; Blay, M.; Pujadas, G.; Ardevol, A.; Salvado, M. J.; Blade, C.; Arola, L.; Fernandez-Larrea, J. *J. Nutr. Biochem.* **2011**, *22*, 153–163.
401. Wren, A. F.; Cleary, M.; Frantz, C.; Melton, S.; Norris, L. *J. Agric. Food Chem.* **2002**, *50*, 2180–2192.
402. Brune, M.; Rossander, L.; Hallberg, L. *Eur. J. Clin. Nutr.* **1989**, *43*, 547–557.
403. Hu, M. *Mol. Pharm.* **2007**, *4*, 803–806.
404. D’Archivio, M.; Filesi, C.; Vari, R.; Scazzocchio, B.; Masella, R. *Int. J. Mol. Sci.* **2010**, *11*, 1321–1342.
405. Deprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A. *J. Nutr.* **2000**, *130*, 2733–2738.
406. Manach, C.; Morand, C.; Texier, O.; Favier, M. L.; Agullo, G.; Demigne, C.; Regerat, F.; Remesy, C. *J. Nutr.* **1995**, *125*, 1911–1922.
407. Janisch, K. M.; Williamson, G.; Needs, P.; Plumb, G. W. *Free Radical Res.* **2004**, *38*, 877–884.
408. Silberberg, M.; Morand, C.; Mathevon, T.; Besson, C.; Manach, C.; Scalbert, A.; Remesy, C. *Eur. J. Nutr.* **2006**, *45*, 88–96.

409. Yurdakul, S.; Ozben, B.; Bilge, A. K.; Turkoglu, U. M.; Arkaya, S.; Nisanci, Y. *J. Invest. Med.* **2008**, *56*, 925–930.
410. Lee, S. H.; Blair, I. A. *Trends Cardiovasc. Med.* **2001**, *11*, 148–155.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch005

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Chapter 6

DNA-Protective Mechanisms of Glutathione Intervention in Catechol-Mediated Oxidative DNA Damage in the Presence of Copper(II) Ions

Maria Hepel,* Magdalena Stobiecka, Janet Peachey, and Jeremiah Miller

Department of Chemistry, State University of New York at Potsdam, Potsdam, New York 13676
***E-mail: hepelmr@potsdam.edu**

Small molecules with well defined antioxidant properties have been investigated in view of their different behavior in the presence of copper(II): catechol acting as a pro-oxidant and causing serious DNA damage and glutathione (GSH) showing propensity to inhibit the catechol-mediated DNA damage. To elucidate the mechanisms leading to these different behaviors of the two antioxidants, we have investigated the melting characteristics for a calf thymus DNA and base oxidation under the conditions of catechol/Cu(II) attack. A novel fluorescence method for DNA melting-onset temperature determination, with DAPI fluorescent probe, has been applied. The base oxidation was monitored using pulse voltammetric techniques. The molecular dynamics and quantum mechanical calculations were applied to evaluate pathways of ROS generated by semiquinone and GSH thiyl radicals, interacting with a major groove in a model DNA, to the C8 site in guanine base where the main oxidation takes place. The mechanisms of antioxidant and DNA-protecting behavior of GSH have been evaluated with emphasis placed on the unique properties of GSH-Cu(I) complex and low redox potential of the GSH/GSSG couple that maintains catechol in the reduced state and thus shuts down the initial step of the Fenton cascade. We have found that other antioxidants, including N-acetylcysteine and

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

N-(2-mercapto-propionyl)glycine, exhibit DNA-protecting properties as well, though the protecting efficiency of GSH appears to be superior to other biomolecules. The catechol-induced DNA damage in the presence of Cu(II) ions has been confirmed by gel electrophoresis. The DNA melting characteristics using DAPI probe are proposed as a convenient technique for testing DNA damage and the effectiveness of antioxidants as the damage preventing species. The melting characteristics can also be utilized for the development of new drugs controlling the DNA damage caused by oxidative stress in diseases.

Keywords: glutathione antioxidant; catechol pro-oxidant; reactive oxygen species; DNA melting determination; DAPI fluorescent probe; DNA pulse voltammetry; N-acetylcysteine antioxidant; N-(2-mercapto-propionyl)glycine antioxidant; catechol pulse voltammetry

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Introduction

Oxidative stress is brought upon a biological organism through the endogenous formation of radical species, e.g. reactive oxygen species (ROS), or by an external attack of strongly oxidizing agents. The oxidative stress results in serious damage of DNA, proteins, and lipids (*1*) and has been implicated in many common diseases (*2*), such as diabetes (*3*, *4*), Alzheimer's (*5–7*), Parkinson's (*8–10*), cardiovascular (*11–14*), acute renal failure (*15*, *16*), cancer (*17*, *18*), Down syndrome (*19*, *20*), and autism (*21*, *22*). The critical role of ROS in many biological processes including aging, mutation, and carcinogenesis cannot be overestimated (*23*, *24*). Recently, one particular source of ROS generation has attracted considerable attention: the pathway in which metal ions, such as Fe^{3+} or Cu^{2+}, are reduced by an organic reductant, followed by the catalytic formation of H_2O_2, $O_2^{\bullet -}$, and $HO^{\bullet}$ in a reaction catalyzed by transition metal ions. We refer to this pathway as the *Fenton cascade*. In this chapter, we present results of investigations of catechol-mediated DNA damage in the presence of Cu(II) ions, with the main focus on the elucidation of the mechanism of different behaviors of two antioxidant species, catechol and glutathione. Catechol appears to be very efficient in acting as a pro-oxidant while GSH maintains its antioxidant role (*25–27*) and prevents DNA damage. The high concentration of Cu(II) reported recently in children with the end-stage renal disease results in prevalent lipid peroxidation and fatal kidney failure (*28*). Therefore, developing a therapy with antioxidants diminishing the ROS generation is of primary importance.

Catechol is a well known cytotoxic, immunotoxic, and genotoxic metabolite of benzene (*29–31*), smoked products, and some drugs, for instance aspirin (*32*). It occurs naturally in foods (including crude beet sugar, onions, smoked fish). Despite of the toxicity, catechol has been widely used in industrial synthesis (*33*) and in pharmaceutical formulations as a preservative (*34*). Upon binding

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

covalently to proteins and DNA, catechol may induce inhibition of cell replication or carcinogenesis (*33*). The oxidation of catechol leads to the formation of semiquinone radicals, which are able to bind to –SH groups in proteins and to $–NH_2$ groups in nucleic acids (*35*), with the macromolecules being inactivated in the process (*36*). A correlation of polyphenol antioxidizing efficiency for preventing DNA damage by binding to iron has been proposed by Brumaghim et al. (*37*). The studies of DNA oxidation and strand scission by reduction of organic compounds in the presence of Fe(III) or Cu(II) (*38*, *39*) have revealed that the oxidation of nitrogen bases is caused by $HO^{\bullet}$ radicals generated in Fenton cascade while the strand breakage is due to either ferryl oxocomplex or Cu(I)-hydroperoxyl complex generated in ROS formation reaction sequences. It has been found that antioxidant-mediated DNA damage in the presence of Cu(II) is more extensive than that in the presence of Fe(II) in a classical Fenton process (*38*).

The electrochemical reactions of catechol electrooxidation to semiquinone and quinone, and back reduction to catechol are fast reactions (*40*). Therefore, catechol can act either as an antioxidant or as a pro-oxidant. Due to the redox cycling, it may inactivate or damage macromolecules such as DNA or proteins (*30*). Bukowska and Kowalska have found that phenol and catechol may induce hemolytic changes in human erythrocytes (*41*).

The catechol moiety is common in many biocompounds (e.g. dopamine, estrogen, etc.). Some compounds with catechol moiety have been considered for therapeutic applications (*29*), for instance, 6-hydroxydopamine has been found to influence the rat's nigrostriatal pathway, which is of key importance in Parkinson's disease (*42*). Green tea catechin polyphenols are considered some of the most potent chemopreventors (*43*) able to reduce the risk of common cancers, including colon, gastric, mammary, liver, lung, and skin. Other antioxidant catechol derivatives, such as 4-methylcatechol and caffeic acid, are carcinogenic (*43*) and induce squamous cell carcinoma or adenocarcinoma. The 4-hydroxyestradiols ($4\text{-OHE}_1(E_2)$), metabolites of estrogen, can form adducts with DNA, upon oxidation of their catechol moiety to quinone (*44*), binding strongly to adenine and guanine. Dissociation of the adduct leaves apurinic sites in DNA and leads to the initiation of cancer (*44*, *45*). The catechol estrogen $4\text{-OHE}_1(E_2)$ is carcinogenic forming depurinating adducts in human DNA (*46*). The synthetic estrogen, hexestrol, also forms depurinating DNA sites due to its catechol moiety (*47*). The common neurotransmitter dopamine (4-(2-aminoethyl)benzene-1,2-diol) has been found to change DNA conformation and generate ROS in the presence of Cu(II) ions (*48*) and it may be involved in metal-induced genotoxicity.

GSH is a ubiquitous antioxidant present in all eukaryotic organisms. However, in the presence of Fe(III), GSH has been found to cause DNA damage at all nucleotides, with strongest damage inflicted on guanine (*49*). Therefore, both antioxidants, GSH and catechol, can induce DNA damage under certain conditions. Catechol has been found to decrease the level of GSH in erythrocytes by 73 %, most likely due to the ROS generation (*41*).

In this study, we present results of investigations of pro-oxidant properties of catechol in the presence of Cu^{2+} ions and its participation in the oxidative DNA damage. An elevated concentration of Cu(II) ($> 20\ \mu M$) has recently been found in acute kidney failure in children (*28*) and associated with the increased ROS generation. We show the utility of a novel fluorescent probe method with a DAPI probe and pulse voltammetry to evaluate catechol-induced DNA damage. The mechanistic considerations of the different behaviors of the antioxidants, catechol and GSH, in the presence of Cu(II) ions, have been presented. The gel electrophoresis has also been applied to corroborate the DNA damage observed in fluorescence measurements. We propose to utilize the characterization of DNA melting with DAPI fluorescent probe as a convenient tool to investigate DNA damage and to develop new drugs that can intervene in the ROS generation process in case of critical diseases, such as the children's acute renal failure and others.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

2. Experimental

2.1. Chemicals

Catechol was purchased from Fisher Scientific Company (Pittsburgh, PA, U.S.A.). Reduced L-glutathione (GSH), deoxyribonucleic acid from calf thymus, sodium salt (ctDNA) as lyophilized powder and fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.). The dsDNA stock solution (1 mg/mL) was prepared at least 24 h before measurements. TRIS-HCl buffer (0.02 M, pH 7.5) or acetate buffer (0.1 M pH 5.0) were used in fluorimetric or pulse voltammetric measurements, respectively. Solutions were prepared using Millipore (Billerica, MA, U.S.A.) Milli-Q deionized water (conductivity σ = 55 nS/cm).

2.2. Fluorimetric Measurements

The fluorimetric measurements were recorded using LS55 Spectrometer (Perkin Elmer, Waltham, MA, U.S.A.) equipped with 20 kW Xenon light source operating in 8 μs pulsing mode. Pulse width at half height was less than 10 μs. Separate monochromators for the incident beam and the detector beam enabled to use monochromatic radiation with wavelengths from 200 nm to 800 nm with 1 nm resolution. The dual detector system consisted of a photomultiplier tube (PMT) and an avalanche photodiode. Both the excitation and emission slit widths were set to 5.0 nm. Measurements were performed in TRIS-HCl buffer (0.02 M, pH 7.5).

2.3. Electrochemical Measurements

The differential-pulse voltammetric (DPV) measurements were performed with a Bipotentiostat CHI1232B with a three-electrode configuration from CH Instruments (Austin, TX, U.S.A.). Potentials were measured versus the Ag/AgCl reference electrode, obtained from Bioanalytical Systems (BAS) (West Lafayette, U.S.A.) Ag/AgCl wire was placed in the glass tube filed with 3 M NaCl and the tube was closed with vycor plug which protect the Ag/AgCl wire from the direct contact with the sample solution. A Pt wire was used as the counter electrode. The glassy carbon electrode (GCE) with an area of 7.1 mm^2, obtained from Elchema (Potsdam, NY, U.S.A.) was used as working electrode. All DPV measurements were performed in 0.1 M acetate buffer blank solution, pH 5.0. The surface of GCE electrode was polished with 0.05 μm alumina slurry (Coating Service Department, Indianapolis, U.S.A.) on a flat pad and rinsed repeatedly with water to remove any alumina residue. The potential was scanned from 0 V to 1.3 V, with pulse amplitude of 50 mV, pulse width of 70 ms and pulse period of 0.5 s. Subsequent measurements were carried out by polishing the surface of GCE and repeating the assay.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

2.4. Preparation of Damaged DNA Samples

The solutions of double stranded ctDNA with added catechol, copper(II) ions and different concentrations of glutathione were incubated for 16 h to obtain damaged DNA. In DPV measurements, a layer of double-stranded ctDNA–modified electrodes were prepared by covering the freshly polished GCE surface with a 6 μL drop of a ctDNA solution and allowing it to dry. Next, the measurements were performed in 0.1 M acetate buffer. In fluorimetric measurements, the solution of fluorescence dye probe DAPI at the given concentration was added to the solution of damaged DNA and measurements were carried out. In experiments where DNA damage was performed at 37 °C temperature, the solutions were incubated for 1 hour at the proper temperature and then the DAPI dye was added.

2.5. Chemicals for Bacteria Transformation and Growth and for Gel Electrophoresis

The pGLO Bacterial Transformation Kit containing *E. coli* HB101 K–12, lyophilized; Ampicillin, lyophilized, 30 mg; L (+) Arabinose, lyophilized, 600 mg; Transformation solution (50 mM $CaCl_2$, pH 6.1) and Quantum Prep Plasmid Miniprep Kit containing: Cell resuspension solution , cell lysis solution, neutralization solution, Quantum Prep Matrix and Wash buffer were obtained from Bio-Rad (Hercules, CA, U.S.A.). Difco™ LB Agar, Lennox, was obtained from Becton, Dickinson and Company (Sparks, MD, USA). Seakem® Le agarose for gel electrophoresis was obtained from BioWhittaker Molecular Applications (BMA) (Rockland, ME, U.S.A.). TRIS ultra-pure buffer was purchased from MP Biomedicals, LLC (Solon, Ohio, U.S.A.). Boric Acid was obtained from Shelton Scientific, (Shelton, CT, U.S.A.). 1 L of TBE buffer solution (10x) consisted of 121 g TRIS, 55 g boric acid, 7.4 g EDTA and pH was adjuced by HCl to pH = 8.3.

2.6. Agarose Gel Electrophoresis

The agarose gel electrophoresis was performed on 1% agarose using Mini-Gel Horizontal Electrophoresis System (VWR, U.S.A). The electrophoresis buffer tray was filled with a 1x TBE buffer solution and the gels were stained with 1% ethidium bromide (1.5 μL). Samples were loaded in 10 μL increments with 2 μL of

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

6x loading buffer consisting of 5.0 mL glycerol, 25 mg bromophenol blue (BPB) and 4.5 mL distilled H_2O. The gels were then run at 140 V using a power supply, model 500 AccuPower (VWR, U.S.A).

2.7. Molecular Dynamics Simulation and Quantum Mechanical Calculations

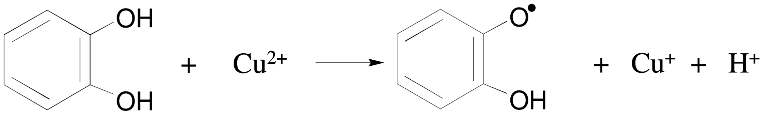
Quantum mechanical calculations of electronic structures for model DNA molecules, catechol, GSH, and their derivatives, as well as the products of interactions between them were performed using modified Hartree-Fock methods with 6-31G* basis set and pseudopotentials, semi-empirical PM3 method, and density functional theory (DFT) with B3LYP functional (*50*, *51*). The molecular dynamics simulations and quantum mechanical calculations were carried out using procedures embedded in Wavefunction (Irvine, CA, U.S.A.) Spartan 6. The electron density and local density of states are expressed in atomic units, where 1 au = 0.52916 Å and 1 au^{-3} = 6.7491 $Å^{-3}$.

3. Results and Discussion

3.1. Detection of DNA Damage by Monitoring DNA Melting Characteristics

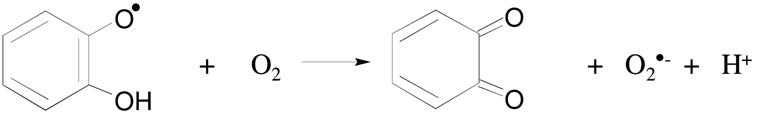
Several types of DNA damage, induced by ROS and other agents, are presented in Scheme 1, including: (1) modifications of DNA bases (adduct formation, base oxidation, base alkylation, base hydrolysis), (2) single and double-strand breaks in the phosphate-deoxyribose DNA backbone, (3) base loss (apurinic sites), (4) damage to the deoxyribose sugar, (5) DNA-protein cross-linkage.

In the case of catechol-mediated DNA damage in the presence of copper ions, the ROS generation results in the oxidation of nitrogen bases and strand scission. The most reactive ROS, causing the oxidation of nucleobases, is the hydroxyl radical $HO^•$, generated in the reaction sequence of the Fenton cascade (1)-(4), defined as follows:



(1)

catechol (Cat) semiquinone radical (SQR)

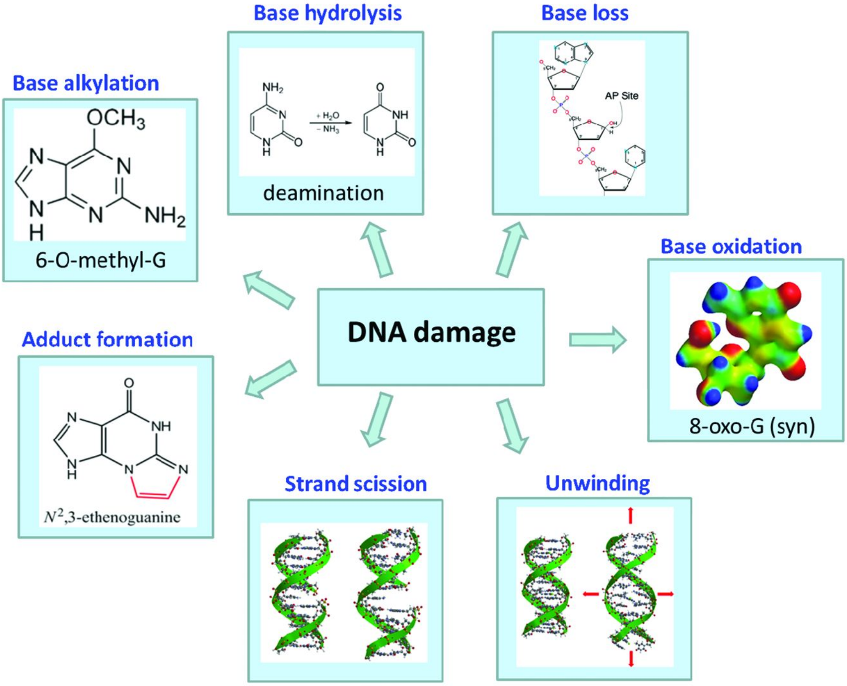


(2)

SQR 1,2-benzoquinone (oBQ)

$$2O_2^{•-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + HO^{\bullet} + OH^{-} \qquad (4)$$


Base hydrolysis
Base loss
Base alkylation
OCH3
N
N
N
N
NH2
H
6-O-methyl-G
NH2
+ H2O
− NH3
NH
O
deamination
AP Site
Base oxidation
8-oxo-G (syn)
DNA damage
Adduct formation
O
NH
N
N
N
H
N2,3-ethenoguanine
Strand scission
Unwinding

Scheme 1. Different kinds of DNA damage caused by oxidative stress, toxicants and alkylating agents.

The oxidation of guanine by $HO^{\bullet}$ occurs at the C8 position, yielding an oxidation product 8-oxoguanine. The interaction of $HO^{\bullet}$ with adenine results in the formation of 8 or 4-,5- hydroxyadenine. The less reactive ROS, including $O_2^{\bullet-}$ and H_2O_2, at their physiological levels and in the absence of Cu(II) or Fe(II), do not inflict any damage to DNA but can serve as a source of other reactive compounds and radicals (*24*). The breakage of DNA strands may occur by a deoxyribose scission in a DNA reaction with copper(I) peroxyl complex Cu(I)OOH (*52*) which forms together with other ROS in the presence of Cu(I). In this study, the assessment of DNA damage was performed using fluorescence spectroscopy with a dye-probe DAPI and electrochemical relaxation technique, differential pulse-voltammetric method.

In fluorescence experiments, the measurements of emission of a DAPIdye following the interactions of catechol with ctDNA at different temperatures were carried out to determine the melting-onset temperature (T_{mo}) as the DNA damage indicator (Figures 1-7). The DAPI probe is a fluorescent dye used in cytochemistry as a stain for binding to dsDNA (*53–55*). The probe binds to DNA specifically at the AT base-pairs in the minor groove without significant perturbation of DNA

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

structure and the complex is stabilized by hydrogen bonding and Van der Waals interactions. DAPI interacts also with DNA by intercalation, mainly into the space between two GC base-pairs or between AT and GC base-pairs (*56–61*). In Figure 1, the fluorescence spectra of a DAPI dye recorded after interactions with DNA and the melting profile for an undamaged ctDNA are presented.

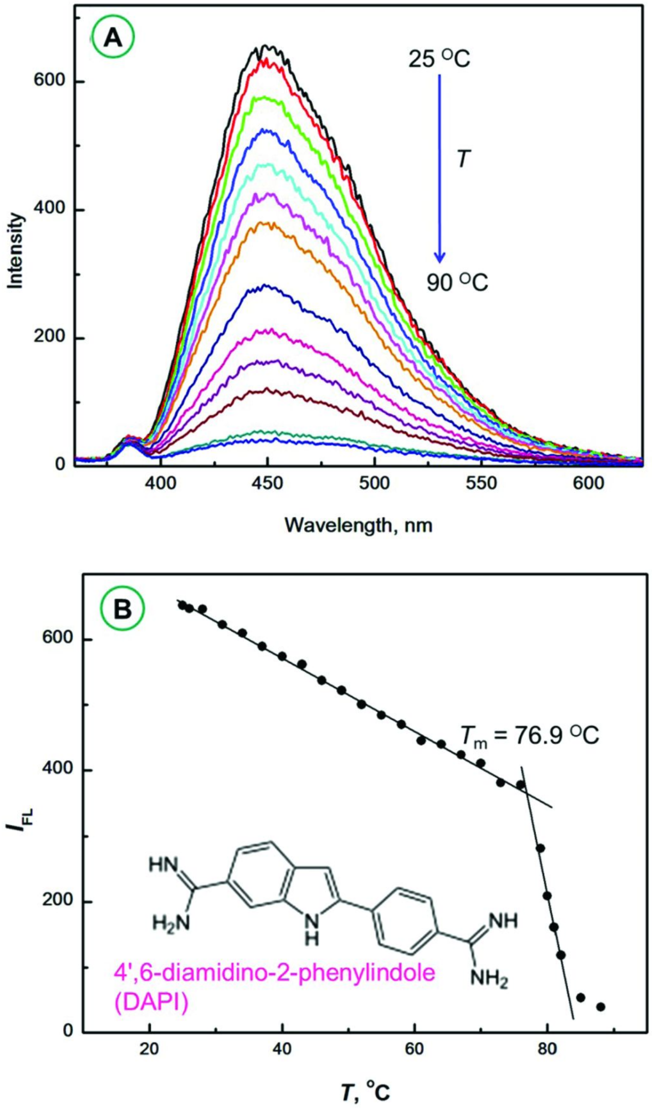


Figure 1. (A) Fluorescence emission spectra of a DAPI-probe interacting with a ctDNA at different temperatures, and (B) dependence of I_{FL} ($\lambda_{em,max}$ = 450 nm) on temperature; C_{ctDNA} = 1.7 μM bp; C_{DAPI} = 167 nM; λ_{ex} = 340 nm.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

We have found that the dsDNA/DAPI fluorescence versus temperature profiles are very characteristic and provide clear interception of two linear temperature dependences below and above the DNA melting-onset. The heating of DNA solutions with DAPI results in a fluorescence to decrease due to the local DNA dehybridization. The melting-onset temperature T_{mo} determined for ctDNA with DAPI-probe was 76.9 °C (Figure 1B). This value is very close to $T_m = 75.5$ °C, probed by Duguid and coworkers (*62*) by differential scanning calorimetry (DSC) for 160 bp ctDNA. We have then performed similar melting-profile measurements for ctDNA in the presence of different agents. We have found that catechol or copper ions alone, do not cause any oxidative DNA damage since the values of T_{mo} obtained for ctDNA in 100 µM catechol solution and for ctDNA in 33.3 µM copper(II) solution were very close to the values of T_{mo} of an undamaged DNA, and they were equal to 78.2 and 77.7 °C, for solutions of catechol and copper(II), respectively. In the presence of 33.3 µM Cu(II), a 100 µM catechol caused a considerable lowering of T_{mo}, with $\Delta T_{mo} = 28.1$ to 50.1 °C (Figure 2) which clearly indicates on a DNA damage.

In the presence of molecular oxygen and heavy metal ions, it is known that catechol can cause DNA strand breaks by scission of the deoxyribose-phosphate backbone (*29*). Our pulse voltammetric experiments confirm that guanine oxidation to 8-oxo-guanine is also taking place while the gel electrophoresis indicates strand cuts in plasmid DNA due to strand scission through the breakage of deoxyribose (vide infra). These results are consistent with other studies (*39*, *63*, *64*).

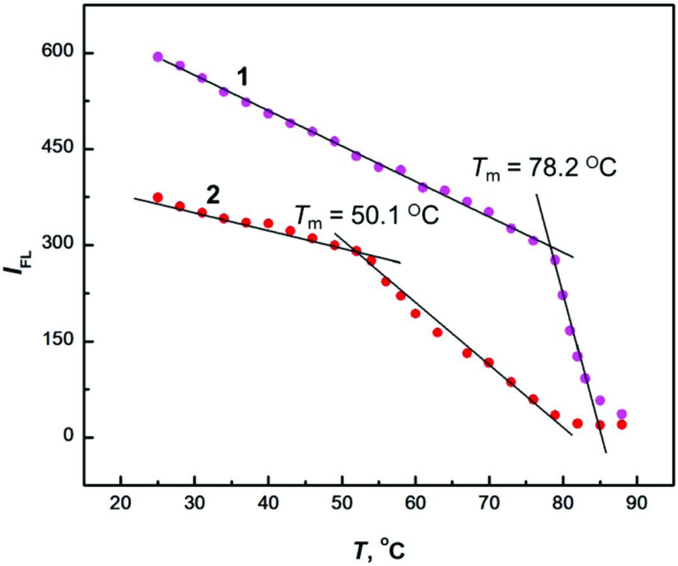


Figure 2. Dependence of DAPI-probe fluorescence on temperature after interactions of ctDNA with: (1) catechol and (2) catechol and Cu^{2+} ions; TRIS-HCl buffer (20 mM, pH = 7.5); C_{ctDNA} = 1.7 µM bp; C_{DAPI} = 167 nM; C_{Cat} = 100 µM; C_{CuSO4}= 33.3 µM; t_{inc} = 16 h.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

In Figure 3, the effect of catechol-mediated DNA damage in the presence of 33 μM Cu(II) ions on DNA melting characteristics is illustrated. It is seen that with the increasing concentration of catechol, C_{Cat}, a more extensive DNA damage is generated which is reflected on the lowering of DNA melting temperature. The relationship between T_{mo} and C_{Cat} was modeled using Boltzmann function (Figure 3B).

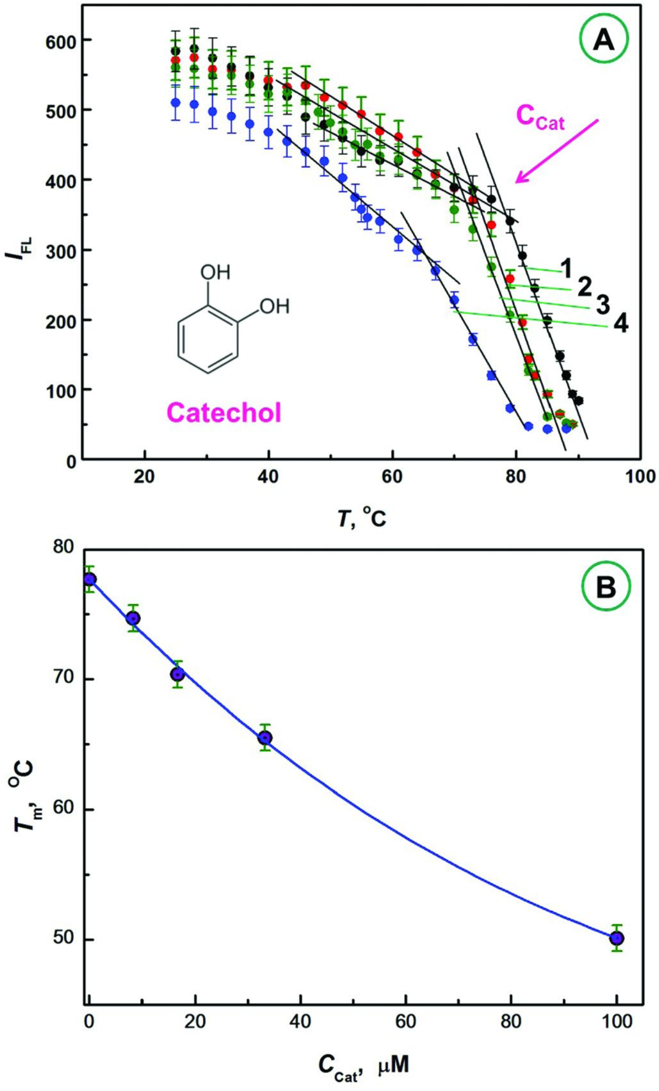


Figure 3. (A) Dependence of DAPI-probe fluorescence on temperature after interactions of ctDNA with Cu^{2+} ions and different concentrations of catechol, in TRIS-HCl buffer (20 mM, pH = 7.5); C_{ctDNA} = 1.7 μM bp; C_{DAPI} = 167 nM; C_{Cat} [μM]: (1) 0, (2) 8.33, (3) 16.67, (4) 33.3; C_{CuSO4}= 33.3 μM; t_{inc} = 1 h, 37 °C; (B) dependence of T_{mo} vs. C_{Cat} using data of Figures 2 and 3A.

3.2. Protective Role of GSH and Related Compounds in Catechol/Cu^{2+}-Mediated DNA Damage

The experiments with DNA protection by antioxidants have been carried out to gain new insights into the mechanism of DNA damage and prevention. In Figure 4, the melting characteristics for a ctDNA in 20 mM TRIS-HCl buffer solution, pH = 7.5, in the presence of catechol and Cu(II), and different concentrations of GSH are presented. In each of the melting profiles recorded in the presence of DAPI, we have observed a transition which reflects denaturation of the duplex into its component single stranded DNA. After addition of 100 μM GSH, we have observed an increase of T_{mo} from T_{mo} = 50.2 °C in the absence of GSH to T_{mo} = 67.2 °C in its presence. Clearly, the addition of GSH has sharply reduced the DNA damage by radicals.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

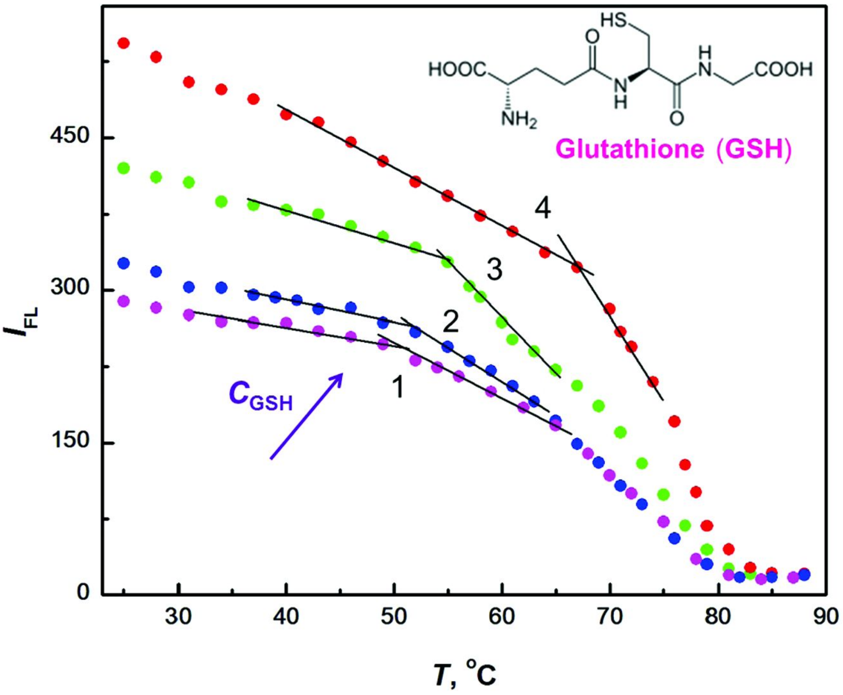


Figure 4. Effect of GSH on temperature dependence of DAPI-probe fluorescence recorded after interaction of ctDNA with catechol and Cu^{2+} ions, in TRIS-HCl buffer (20 mM, pH = 7.5), C_{GSH} [μM]: (1) 20, (2) 33.3, (3) 50, (4) 100. C_{ctDNA} = 1.7 μM bp; C_{DAPI} = 167 nM; C_{CuSO4}= 33.3 μM; C_{Cat} = 100 μM; t_{inc} = 1 h, 37 °C; λ_{ex} = 340 nm; λ_{em} = 450 nm.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Similar experiments have been performed using N-acetylcysteine which is a GSH precursor and is an excellent dietary supplement (*65*) to replenish GSH that has been used in fighting oxidative stress, for instance in the case of dialysis patients. The ctDNA melting characteristics in the presence of damage-inducing catechol and Cu(II), and N-acetylcysteine are presented in Figure 5. It is seen that with increasing concentration of N-acetylcysteine, less DNA damage is observed. Therefore, N-acetylcysteine itself, similar to GSH, shows inhibiting properties for Fenton cascade reactions generating DNA-damaging ROS as well. N-acetylcysteine is produced endogenously and is found in foods. It converts to cysteine by hydrolysis and also delivers GSH by deacetylation. N-acetylcysteine has a sulfhydryl group and is capable of scavenging free radicals (*66*). Recently, the Rogan group has found that N-acetylcysteine and another antioxidant, resveratrol, block the formation of cancer-initiating estrogen-DNA adducts in cells (*45*, *67*).

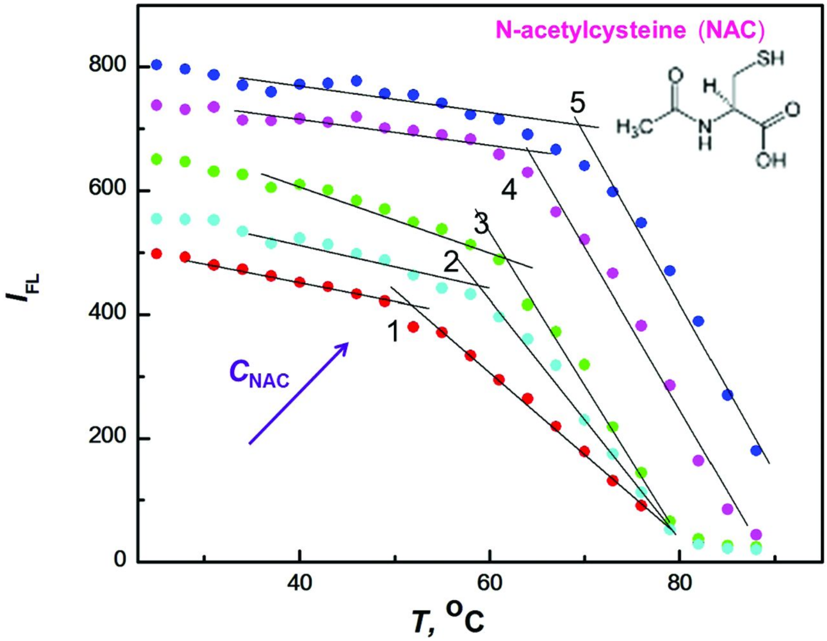


Figure 5. Effect of GSH precursor, N-acetylcysteine (NAC) on temperature dependence of DAPI-probe fluorescence recorded after interaction of ctDNA with catechol and Cu^{2+} ions, in TRIS-HCl buffer (20 mM, pH = 7.5), C_{NAC} [mM]: (1) 0.2, (2) 1, (3) 2, (4) 3, (5) 4. C_{ctDNA} = 1.7 μM bp; C_{DAPI} = 167 nM; C_{Cat} = 100 μM; C_{CuSO4}= 33.3 μM; t_{inc} = 1 h, 37 °C; λ_{ex} = 340 nm; λ_{em} = 450 nm.

To compare the antioxidant concentrations required for an effective prevention of catechol-mediated DNA damage in the presence of Cu(II), we have plotted the dependencies of T_{mo} vs C for GSH and N-acetylcysteine in Figure

6. It is apparent that GSH is far more effective in protecting ctDNA against catechol-induced oxidative damage since the GSH concentration required for 50% DNA protection is *ca.* 100 μM while that for N-acetylcysteine is *ca.* 2.2 mM.

These critical concentrations have been determined graphically from the plot of T_{mo} vs. C obtained for antioxidants, GSH and N-acetylcysteine, presented in Figure 6. The 50% protection level has been calculated using the equation: $T_{mo,50} = T_{mo} - ½(T_{mo} - T_{mo,100})$, where T_{mo} is the melting-onset temperature for an undamaged DNA and $T_{mo,100}$ is the DNA melting-onset temperature for a maximum DNA damage (100%) under the given experimental conditions.

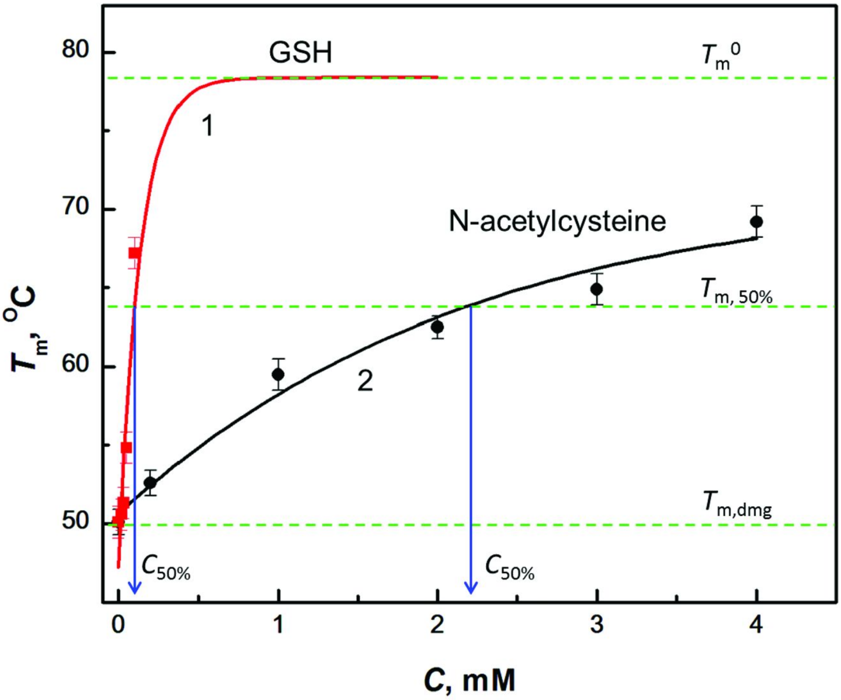


Figure 6. Antioxidant concentration dependence of ctDNA melting-onset temperature for ctDNA subjected to damaging concentration of catechol and Cu(II), for: (1) GSH, (2) N-acetylcysteine. TRIS-HCl buffer (20 mM, pH = 7.5). $C_{ctDNA} = 1.7$ μM bp; $C_{DAPI} = 167$ nM; $C_{Cat} = 100$ μM; $C_{CuSO4} = 33.3$ μM; $t_{inc} = 1$ h, 37 °C; the T_{mo} level: $T_{mo} = 78.2$ °C, has been determined from Figure 2, curve 1, and it is higher than that observed for ctDNA in absence of catechol ($T_{mo} = 76.9$ °C, Figure 1) because of the stabilizing interactions of ctDNA with catechol (in absence of Cu(II)) in the former case.

Among other antioxidants expected to act similar to GSH, we have also tested N-(2-mercapto-propionyl)glycine (MPGly), a thiol related to GSH. The temperature dependence of DAPI-probe fluorescence, recorded after interaction

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

of ctDNA with catechol and Cu^{2+} ions, is presented in Figure 7. MPGly is a condensation product of the reaction of thiolactic acid with glycine and is capable of scavenging free radicals generated in the intracellular space (*68*). MPGly has cardioprotective properties, likely due to prevention of free radicals.

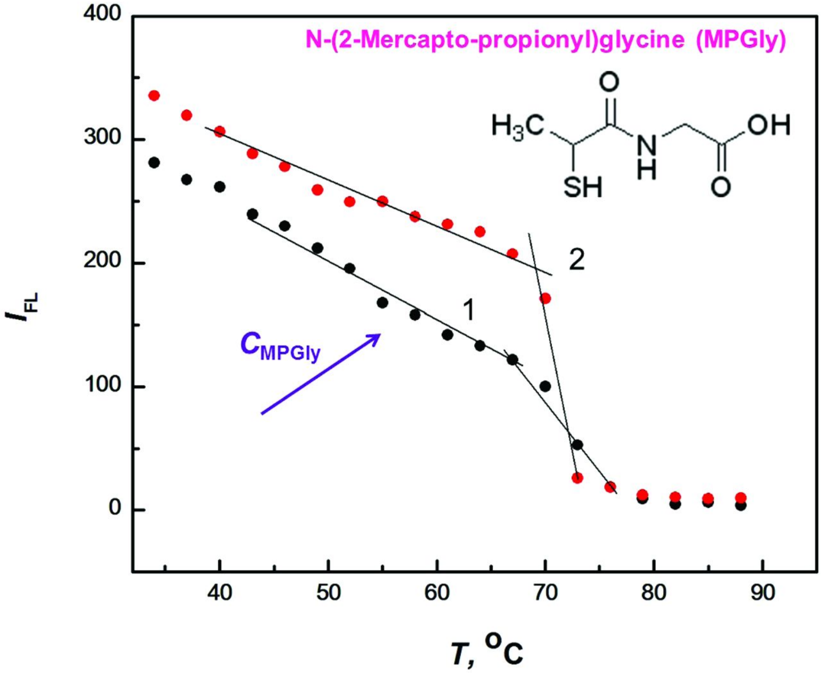


Figure 7. Effect of N-(2-mercapto-propionyl)glycine (MPGly) on temperature dependence of DAPI-probe fluorescence recorded after interaction of ctDNA with catechol and Cu^{2+} ions, in TRIS-HCl buffer (20 mM, pH = 7.5), C_{MPGly} [mM]: (1) 2, (2) 4. C_{ctDNA} = 1.7 µM bp; C_{DAPI} = 167 nM; C_{Cat} = 100 µM C_{CuSO4}= 33.3 µM; t_{inc} = 1 h, 37°C.

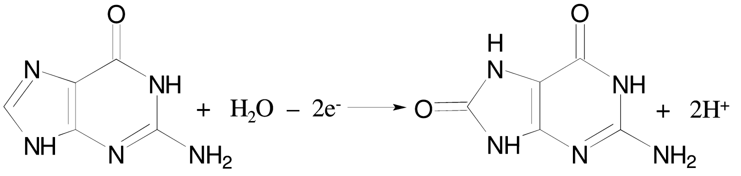
3.3. Detection of DNA Damage Using Voltammetric Monitoring

In order to verify if the base oxidation is occurring in the catechol-mediated DNA damage processes in presence of Cu(II), the electrochemical voltammetric measurements have been performed. In Figure 8A, the differential pulse voltammogram for a double-stranded ctDNA in acetate buffer is presented. The curve shows two anodic peaks corresponding to the oxidation of guanosine (E = 0.88 V, vs. Ag/AgCl) and adenosine (E = 1.11 V) residues in the DNA backbone. The oxidation peak of catechol deposited on GCE surface appears on the voltammetric curve at E = 0.166 V (Figure 8B). The interactions of catechol with DNA and copper(II) ions cause the oxidation peak of catechol to decrease and the peak potential to shift toward more positive potential, E = 0.227 V, with the separation of peaks ΔE = 61 mV. In cyclic voltammetric measurements

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

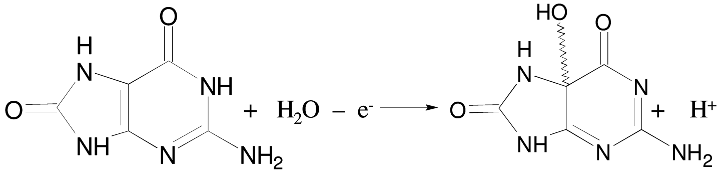
(data not shown), the anodic peak of catechol also shifts toward more positive potentials, from E = 0.23 V to 0.273 V, following the interactions with DNA. It indicates on the increased stability of the reduced form of catechol, most likely due to the adduct formation with ctDNA. Similar results were obtained by Wang and coworkers (*69*) who have detected a DNA damage done by hydroquinone and catechol using an electrochemical DNA biosensor. We have observed additional changes in DPV after incubation of ctDNA with catechol and copper(II). A new peak appeared at E = 0.41 V (Figure 8C). Among the four nucleobases in DNA chain, the guanine is the most easily oxidized base. The oxidation of guanine, both in electrochemical processing and in reaction with $HO^{\bullet}$ radicals in vivo, leads to the formation of 8-oxoguanine (oxoGua) (*70–72*). The electrochemical oxidation of guanine proceeds with the exchange of two electrons and a voltammetric peak is observed at $E_{pa,5}$ = 0.88 V (reaction 5):



(5)

guanine 8-oxo-7-hydroguanine (oxoGua)

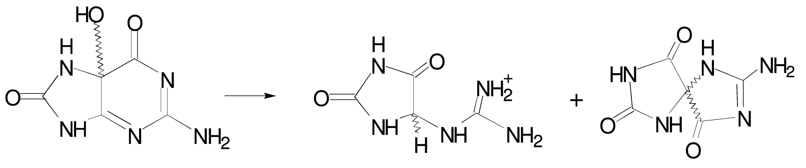
The oxoGua is oxidizable at a potential $E_{pa,6}$ = 0.41 V (reaction (6)), lower by 0.47 V than that for the guanine base itself, consistent with earlier studies (*70*, *71*):



(6)

oxoGua 5-OH-oxoGua

and the final oxidation products include guanidinohydantoin and spiroiminodihydantoin (*72*) and deoxyribosylurea (*73*):



(7)

5-OH-oxoGua guanidinohydantoin spiroiminodihydantoin

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

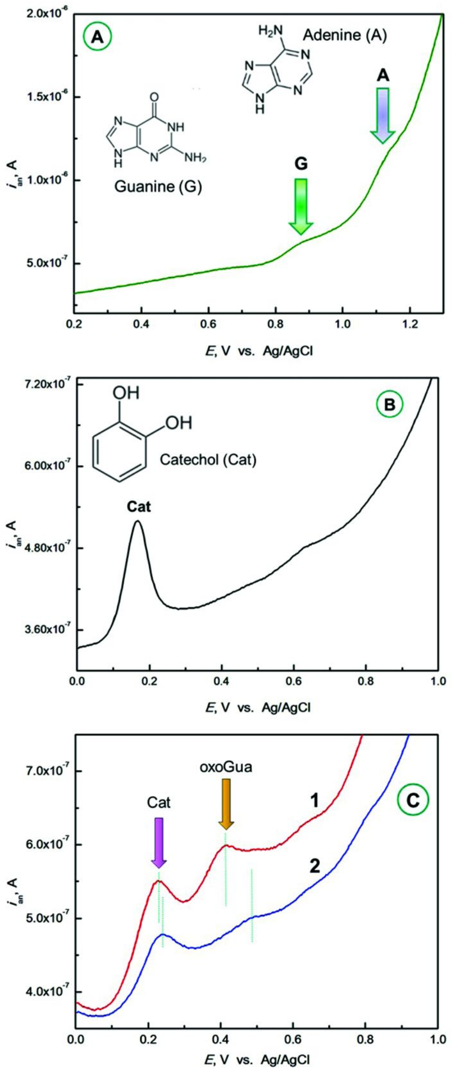


Figure 8. Differential pulse voltammograms (DPV) for: (A) calf thymus DNA (ctDNA), (B) catechol, (C) ctDNA damaged by catechol-mediated oxidation in the presence of Cu^{2+} ions: (1) without GSH and (2) incubated with GSH; C_{ctDNA} = 9.9 μM bp; C_{Cat} = 100 μM; C_{CuSO4}= 33.3 μM; C_{GSH} = 33.3 μM; incubation time t_{inc} = 16 h; amplitude: 50 mV, pulse width: 70 ms, pulse period 0.5 s; medium: acetate buffer (0.1 M, pH 5). Cat - catechol, oxoGua - oxoguanine, G - guanine, A - adenine.

Because in an undamaged DNA there is no oxoGua, therefore the new oxoGua peak observed in DPV in Figure 8C must be due to the oxoGua formed during the oxidative DNA damage. It indicates that catechol and copper(II) induce oxidative damage to DNA. The amplitude of the oxoGua peak decreased after the addition of increased concentrations of GSH to the solution containing copper(II), catechol and ctDNA (Figures 9 and 8C). These results demonstrate that it is possible to detect and quantify the effect of GSH antioxidant action and protection of DNA in simple experiments.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

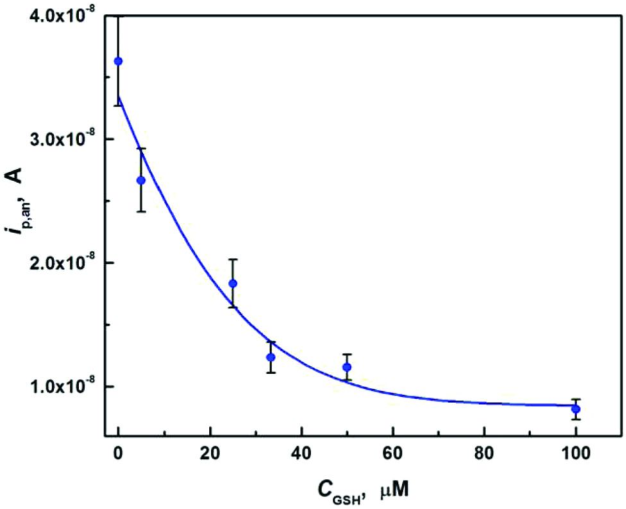


Figure 9. Dependence of DPV peak current for oxoGua oxidation on GSH concentration C_{GSH}, recorded in acetate buffer (0.1 M, pH = 5); C_{ctDNA} = 9.9 μM bp; C_{Cat} = 100 μM; C_{CuSO4}= 33.3 μM; t_{inc} = 16 h; amplitude 50 mV, pulse width 70 ms, pulse period 0.5 s.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

3.4. Molecular Dynamics Simulation of the Interaction of Catecholic Species with Major Groove Nucleobases

The results of our fluorescence measurements indicate that the DNA melting temperature increases upon the addition of catechol to a DNA solution in absence of Cu(II) ions. This clearly demonstrates the ability of catechol to interact with DNA. While the nature of this interaction is not known, its impact on the DNA damage in the presence of Cu(II) may be of primary importance since it may determine the distance the ROS generated at a catechol site needs to travel to the DNA sites prone to oxidative damage. In view of the short lifetime of HO• radicals, which are the main DNA-damaging ROS, this distance is critical to the DNA damage efficiency. To explore further the nature of the interactions of catechol and semiquinone radical with DNA and to calculate the distance between a ROS-generating site and a DNA site to be oxidized, we have performed molecular dynamics and quantum mechanical calculations of the electronic structures of DNA adducts with catechol and GSH, and also with catechol semiquinone radical and GSH thiyl radical.

Catechol, and the more reactive semiquinone radical, can interact with DNA from both the minor groove and from the major groove side. In Figure 10, the molecular dynamics (MD) simulations of the formation of a hydrogen bond between semiquinone radical and guanine of the major groove GC base-pair, is illustrated. The electronic structures were obtained by quantum mechanical (QM) calculations using Spartan 6 software. The hydrogen bond shown here with the dashed line originates from a OH group of semiquinone and binds to nitrogen N7 of guanine. The distance from the ROS-generating C-O• group in semiquinone radical center (SQRC) to C8 atom of guanine in the GC nucleobases in the major groove in DNA is $d_{\mathrm{C8(G)\text{-}SQRC}} = 0.27$ nm. This distance can be compared with that from the S• group in a GS• thiyl radical center (GSRC) to the major groove, presented in Figure 11, which is $dC8(G)\text{-}GSRC = 1.31$ nm. Because of the round path for the HO• diffusion, we can assume $d_{\mathrm{C8(G)\text{-}GSRC}} = 1.31*(\pi/2) = 2.1$ nm. Much smaller distance between nucleobases and the ROS-generating center for semiquinone radical than the respective distance for GS• radical has a consequence in a much greater DNA damage by catechol-generated ROS since there is a greater chance of survival for ROS formed in direct vicinity of nucleobases. The short-lived HO• radicals are implicated here in the oxidation of nucleobases where guanine is oxidized to oxoGua.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

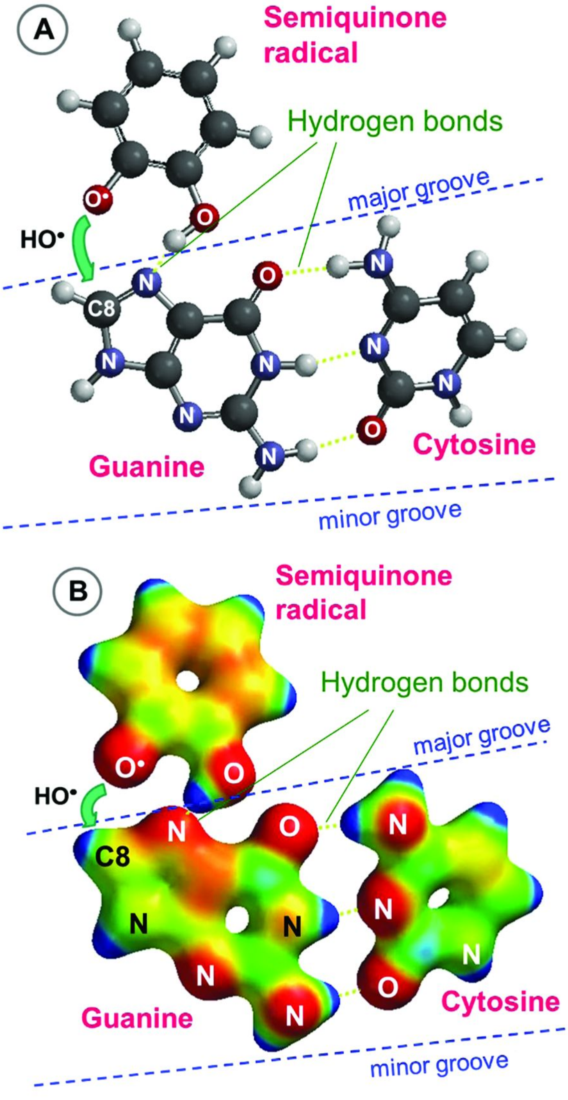

A
Semiquinone radical
Hydrogen bonds
major groove
O•
O
HO•
C8
N
N
O
N
N
N
N
N
O
Guanine
Cytosine
minor groove
B
Semiquinone radical
Hydrogen bonds
major groove
O•
O
HO•
N
O
N
C8
N
N
N
O
N
N
N
Guanine
Cytosine
minor groove

Figure 10. Electronic structure of semiquinone radical interacting with the major-groove GC nucleobase-pair, showing the stabilizing hydrogen bond and indicating the short distance between the ROS-generating radical center and C8 atom of guanine undergoing oxidation to 8-oxoguanine; (A) structure, (B) electron density surface for ρ = 0.08 au^{-3}, mapped with electrostatic potential (color-coded, from low - red, to high - blue).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

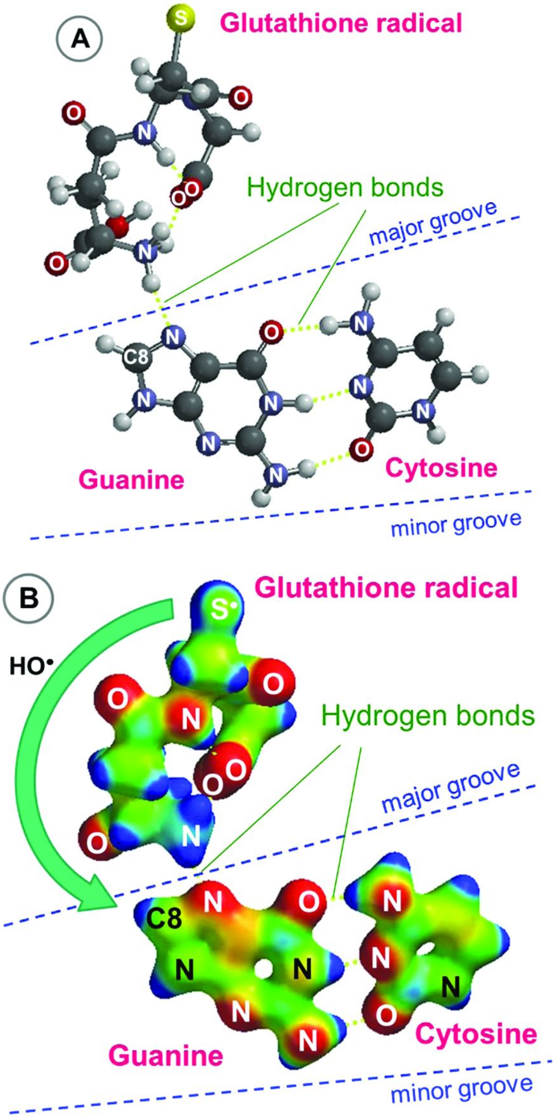

A
S
Glutathione radical
O
N
O
O
O
N
O
Hydrogen bonds
major groove
N
C8
N
O
N
N
N
N
N
O
N
Guanine
Cytosine
minor groove
B
Glutathione radical
S•
HO•
O
O
N
O
O
O
N
Hydrogen bonds
major groove
C8
N
O
N
N
N
N
N
N
O
N
Guanine
Cytosine
minor groove

Figure 11. Electronic structure of glutathione thiyl radical interacting with the major-groove GC nucleobase-pair, showing the stabilizing hydrogen bond and indicating the distance between the ROS-generating radical center and C8 atom of guanine undergoing oxidation to 8-oxoguanine; (A) structure, (B) electron density surface for $\rho = 0.08$ au^{-3}, mapped with electrostatic potential(color-coded, from low - red, to high - blue).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

3.5. Control Experiments with Gel Electrophoresis

The catechol-induced ROS generation in the presence of Cu(II) and the resulting DNA damage can be demonstrated readily in control experiments with agarose gel electrophoresis. For this purpose, we have used a model plasmid DNA.

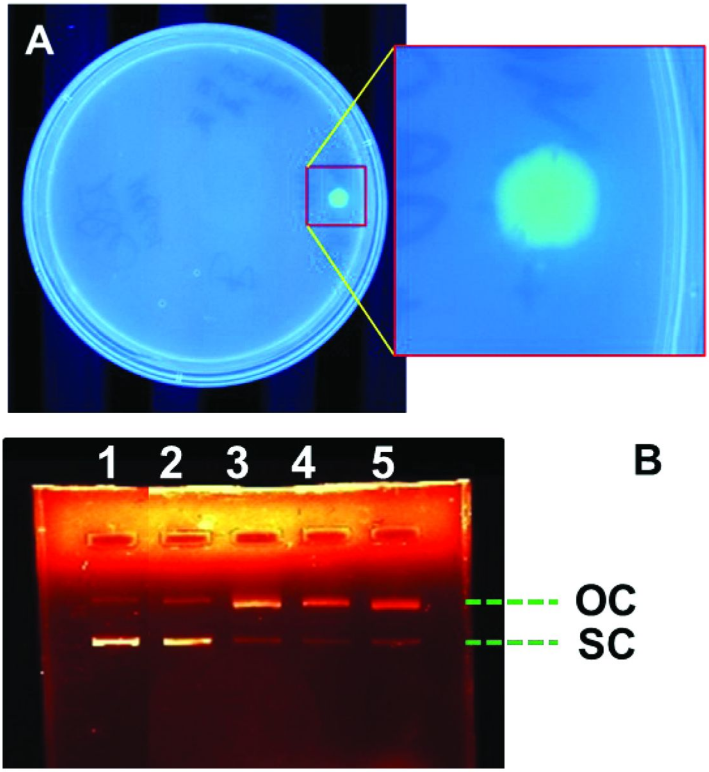


Figure 12. (A) pGLO plasmid DNA visualized under UV light. (B) Agarose gel electrophoretic patterns of plasmid pGLO DNA after interactions with Cu(II) ions and catechol; lane 1: control DNA (plasmid only); (lanes 2-5) plasmid with 0.83 mM Cu(II) and varying concentration of catechol, C_{Cat} [mM]: (2) 0.83, (3) 2.083, (4) 4.17, (5) 8.33; SC - undamaged DNA (supercoiled), OC - nicked DNA (open circular); electrophoresis voltage: 140 V; TBE buffer.

A pGLO plasmid was first inserted into the *Escherichia Coli* bacteria, HB101 K-12 strain, and bacteria were grown on a plate containing sugar arabinose and an antibiotic ampicillin, both included in the agar nutrient. Figure 12A shows a

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

growing colony of the transformed *E. Coli* cells. After extraction and purification, the pGLO plasmid was treated with catechol and Cu(II) and products of the DNA damage by ROS were analyzed using gel electrophoresis (Figure 12B). In the agarose-gel image, the original pGLO plasmid is represented by the fast moving band of a supercoiled DNA form (Figure 12B, band SC). The DNA damage was assessed by measuring the conversion of supercoiled pGLO-plasmid DNA to open circular (OC) form. After incubation of the plasmid with Cu(II) (830 µM) and different concentrations of catechol, the intensity of the supercoiled plasmid band decreased and a new slow moving band of open circular (nicked) DNA form has appeared in the gel image (Figure 12, band OC). The intensity of this band increases with increasing concentration of catechol (not shown), consistent with our measurements of DNA-melting temperature changes. Further increase of the catechol concentration resulted in OC DNA cuts into smaller fast-migrating DNA pieces that disappear quickly from the field of view during the electrophoresis experiment. This is evidenced in Figure 12, lanes 3-4, where the intensity of the OC band decreases with increasing C_{Cat}. At high $C_{Cat}/C_{Cu(II)}$ ratio (lane 5), the intensity of the OC band increases due to the anti-oxidant effect of catechol molecules that are not associated with Cu(II) ions. No damage of DNA was observed in the presence of copper ions and catechol alone (data not shown).

In summary, the gel electrophoresis experiments confirm the DNA damaging power of catechol-induced ROS, generated in the presence of Cu(II) ions, which we have also observed in fluorescence measurements by monitoring DNA melting characteristics.

3.6. Mechanism of GSH Protection against DNA Damage

The oxidation of catechol under physiological pH condition leads to formation of semiquinone radicals and quinone (*29*). In the oxidation of catechol by molecular oxygen in the presence of heavy metal ions, one electron is transferred from catechol to oxygen leading to the formation of a superoxide ($O_2^{\bullet-}$). In the presence of heavy metal ions and catechol, the superoxide is reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). In the absence of catalysis by heavy metal ions, catechol itself can generate H_2O_2 but not O_2^- and $HO^{\bullet}$. According to Baretto et al. (*29*), the DNA strand breaks are caused by the reaction of catechol with Cu^{2+} ions yielding Cu^+ ions and semiquinone radicals. In the following reaction, catalyzed by copper, molecular oxygen is reduced to form $O_2^{\bullet-}$ and H_2O_2. The DNA strand breaks are induced by a DNA-copper peroxyl complex [DNA-Cu(I)OOH] (*63*, *74*), releasing hydroxyl radicals in the vicinity of the DNA (Figure 13).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

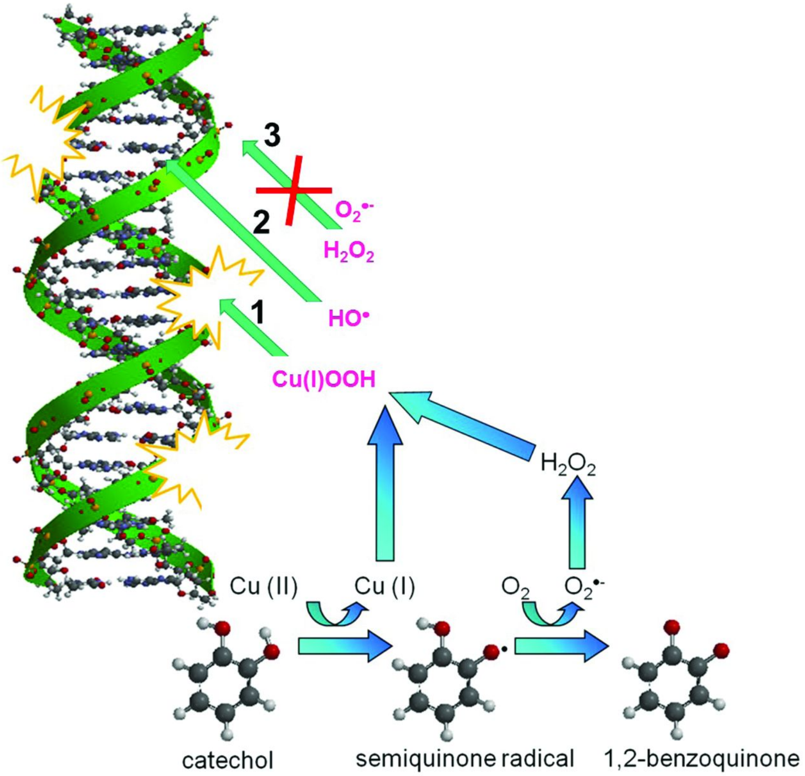


Figure 13. Mechanism of catechol-mediated oxidative DNA damage in the presence of Cu^{2+} ions: (1) strand breaks caused by Cu(I)-hydroperoxyl complex, (2) guanine oxidation by $HO^{\bullet}$ radical, (3) no damage by H_2O_2 and $O_2^{\bullet-}$.

The DNA damage caused by catechol in the presence of Cu(II) can be largely prevented by adding GSH to a DNA solution, as shown in Figure 4. Evidently, GSH breaks the reaction sequence leading to the DNA damage. The plausible explanation is that GSH forms complexes with Cu^+ (log $\beta'_{122} = 38.8$ for $[Cu^+(HL^{2-})_2]^{3-}$ (*75*) where H_3L stands for the neutral glutathione) and prevents the catalytic formation of ROS (*76*), thereby protecting the DNA against the oxidative damage. However, one can argue that complexing of GSH with Cu^{2+} is equally important since it decreases the redox potential of the couple Cu(II)/Cu(I) (*26*), though the complexation of Cu(II) is short-lasting when the ratio of GSH/Cu(II) is 3:1 or higher (*77*) and leads to the formation of a Cu(I)-GSH complex through the reactions:

$$Cu^{2+} + 2\ GSH^- = Cu^{2+}(GS^{2-})_2 + 2H^+ \tag{8}$$

$$Cu^{2+}(GS^{2-})_2 + GSH^- = Cu^+(GS^{2-})_2 + GS^{\bullet-} + H^+ \tag{9}$$

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

where GSH^- symbol indicates the charge in neutral solutions, pH = 7, and shows explicitly the hydrogen bound to the SH group (pK_a values for GSH are: pK_1 = 2.04 (glutamate –COOH), pK_2 = 3.4 (glycine –COOH), pK_3 = 8.72 (–SH group), pK_4 = 9.49 (–NH_2 group)). Only by adding more strongly complexing Hg^{2+} cations, the reduction of Cu(II) by GSH can be prevented (*78*). Similar mechanism has been recently proposed by Brumaghim for metal-mediated DNA damage studied by gel electrophoresis (*76*, *79*), although the role of Cu^{2+} complexation by GSH was not considered.

By comparing the two antioxidants, catechol and GSH, we have found that there is a large difference in the distance between $d_{\mathrm{C8(G)\text{-}SQRC}}$ and *dC8(G)-GSRC* for semiquinone radical and glutathione thiyl radical interacting with DNA (0.27 and 2.1 nm, respectively). Because of the short lifetime of $HO^•$ radicals ($\tau_{1/2}$ = 4.5×10^{-9} s (*80*)), this translates to much lower chance of survival for $HO^•$ radicals generated at $GS^•$ radical center to reach the guanine C8 atom where the primary oxidative damage (to oxoguanine) is taking place. Therefore, the transport of ROS from semiquinone is more efficient than that from $GS^•$ thiyl radical. However, even at the distance of 2.1 nm, part of ROS formed at $GS^•$ is still active and therefore the complexation effects of GSH to Cu(I) and Cu(II) must play an important part in the elucidation of antioxidant properties of catechol and GSH. This is supported by the following considerations. The mean free path d_{OH} for $HO^•$ diffusion can be estimated from the diffusion coefficient of $HO^•$ radicals: $D_{\mathrm{OH}} = 2.3\times10^{-5}$ cm^2 s^{-1} (*81*) and $\tau_{1/2}$ as follows:

$$d_{\mathrm{OH}} = \sqrt{D\tau_{1/2}} = 3.2\,\mathrm{nm} \tag{10}$$

Since the $HO^•$ flux Φ is inversely proportional to distance:

$$\Phi = \frac{DC_{\mathrm{OH}}}{\sqrt{\pi Dt}} = \frac{DC_{\mathrm{OH}}}{\Delta x} \tag{11}$$

where C_{OH} is the concentration of $HO^•$ at the source and Δx is the characteristic diffusion distance. Hence, the ratio of $HO^•$ fluxes from SQRC and from GCRC to the C8 guanine atom is:

$$\frac{\Phi_{\mathrm{SQRC}}}{\Phi_{\mathrm{GSRC}}} = \frac{\Delta x_{\mathrm{GSRC}}}{\Delta x_{\mathrm{SQRC}}} = \frac{2.1}{0.27} \approx 8 \tag{12}$$

Therefore, approximately an order of magnitude higher $HO^•$ flux is expected from the semiquinone than from $GS^•$ thiyl radicals attached to DNA via hydrogen bonds, provided that the rates of $HO^•$ generation are equal. A mild GSH-mediated DNA damage was observed in the presence of Cu(II) ions but not in the presence of Fe(III) ions (*38*). The $HO^•$ flux from the $GS^•$ radical site is further diminished due to the inhibition of catalytic action of Cu(I) ions through the complex formation with GSH.

Further insights into the different behavior of catechol and GSH can be gained by comparing the oxidizing power of GSH and catechol. The redox potential of GSSG/GSH couple is given by:

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

$$\mathrm{GSSG} + 2\mathrm{H}^{+} + 2\mathrm{e}^{-} = 2\ \mathrm{GSH} \tag{13}$$

$$E = E^{0'}_{\mathrm{GSSG/GSH,pH=7}} + \frac{2.303RT}{nF}\log\left(\frac{C_{\mathrm{GSSG}}}{C^{2}_{\mathrm{GSH}}}\right) - \frac{2.303RT}{F}(\mathrm{pH} - 7) \tag{14}$$

where the relative standard potential at pH = 7 is (*82*) (*83*):

$$E^{0'}_{\mathrm{GSSG/GSH,pH=7}} = -0.240\mathrm{V} \tag{15}$$

n = 2, R is the gas constant, T is the absolute temperature, and the Nernstian slope 2.302RT/F = 0.05916 V at 25 °C. The redox potential for GSSG/GSH couple is lower than the redox potential of oBQ/Cat given by:

$$\mathrm{oBQ} + 2\mathrm{H}+ + 2\mathrm{e}- = \mathrm{Cat} \tag{16}$$

$$E = E^{0'}_{\mathrm{oBQ/Cat,pH=7}} + \frac{2.303RT}{nF}\log\left(\frac{C_{\mathrm{oBQ}}}{C_{\mathrm{Cat}}}\right) - \frac{2.303RT}{F}(\mathrm{pH} - 7) \tag{17}$$

with n = 2 and the relative standard potential equal to (*40*):

$$E^{0'}_{\mathrm{oBQ/Cat,pH=7}} = +0.380\mathrm{V} \tag{18}$$

Therefore, the antioxidant power of GSH is much stronger than that of catechol. Despite this, GSH is unable to reduce enough Cu(II) ions and drive the Fenton cascade efficiently. According to Brumaghim (*79*, *84*), sulfur and selenium compounds could inhibit copper(II)-mediated oxidative damage through Cu^{+} coordination. However, if only Cu(I) coordination took place, then the redox potential of the couple Cu(II)/Cu(I) would increase considerably threatening oxidation of guanine even without ROS. This is not likely to happen because Cu^{2+} is also complexed by GSH. The complex $Cu^{II}(SG)_2$ is short-lived and is reduced to a complex in which copper ions are effectively present as Cu(I) ions. This complex is coordinatively saturated and appears to have very low activity toward ROS generation, especially at high concentration ratio of GSH:Cu(II). Hence, the first intervention of GSH is to down regulate kinetics of the ROS generation process. The implications of this mechanism are inordinate: the complexing power of GSH and the unique properties of the reduced copper complex Cu(I)-GSH dominate over its reducing power making it incapable of generating ROS by the Fenton-cascade, in contrast to the pro-oxidant activity of catechol which also forms complexes with copper ions but their stability constants (log $\beta'_{112} = 13.6$ for $[Cu^{+}(H_2L)]$, log $\beta'_{122} = 24.9$ for $[Cu^{+}(H_2L)_2]$, where log $\beta'_{2} = 22.25$ for H_2L representing catechol (*85*)) are lower than those for GSH complexes. Thus, GSH can inhibit catechol-mediated ROS generation. On the other hand, the lower redox potential of GSH keeps catechol in the reduced state which breaks the Fenton cascade at the very first step.

We have to emphasize that the strong antioxidant properties of GSH expressed by its low redox potential are one of the most important features of GSH action in preventing catechol-mediated DNA damage in the presence of Cu(II) ions. The intervention of GSH results in breaking the Fenton cascade in the first reaction of the cascade where Cu(II) is reduced to Cu(I) by catechol. Here GSH prevents catechol from undergoing oxidation to semiquinone radical to drive Cu(II) reduction to Cu(I) which stops the following reactions of ROS generation.

4. Conclusions

We have demonstrated that the antioxidant catechol causes considerable oxidative DNA damage in the presence of Cu(II) ions. We have presented a new evidence that GSH and the GSH-precursor, N-acetylcysteine, can largely prevent the catechol-mediated DNA damage by employing a novel experimental approach, in which the DNA damage was assessed by measuring changes in the DNA melting-onset temperature, determined by fluorescence measurements with a dye-probe DAPI. The independent confirmation of DNA oxidation was obtained by a voltammetric relaxation method (DPV) and a gel electrophoresis. These techniques enable detecting and quantifying DNA damage. They can be used in assessing the antioxidant properties of compounds for therapeutic treatments of oxidative stress. We have demonstrated that other antioxidants, including N-acetylcysteine and N-(2-mercapto-propionyl)glycine, also exhibit DNA-protecting properties. However, the protecting efficiency of GSH appears to be far superior since the critical antioxidant concentrations required to prevent 50% of the DNA damage by a solution of 100 μM catechol + 33.3 M Cu(II) were 0.1, 2.2 and 2.0 mM, for GSH, N-acetylcysteine and N-(2-mercapto-propionyl)glycine, respectively. The interactions of catechol semiquinone radical and glutathione thiyl radical with DNA have been investigated using molecular dynamics and quantum mechanical calculations, revealing more favorable transport conditions for the damaging $HO^{\bullet}$ diffusion to nucleobases from semiquinone radical centers than from $GS^{\bullet}$ thiyl radical centers. In addition to the differences in $HO^{\bullet}$ transport, favoring more extensive damage to DNA by catechol in the presence of Cu(II), there are two other major factors in different behavior of catechol and GSH: (i) the formation and unique properties of the reduced copper complex with GSH and (ii) the redox potential of GSH, lower than that of catechol, which keeps catechol in its reduced state and breaks the Fenton cascade (1)-(4) that produces the DNA-damaging ROS.

Acknowledgments

This work was partially supported by the U.S. DoD grant No. AS073218. The Authors are indebted to Dr. Jan Trybula for help with gel electrophoresis experiments.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

List of Abbreviations

A - adenine,
AT - adenine-thymine Watson-Crick base-pairs,
Cat - catechol,
ctDNA - deoxyribonucleic acid from calf thymus,
DAPI - fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride,
[DNA-Cu(I)-OOH] - DNA-copper-hydroperoxyl complex;
DPV - differential-pulse voltammetry,
DSC - differential scanning calorimetry,
G - guanine,
GC - guanine–cytosine Watson-Crick base-pairs,
GCE - glassy carbon electrode,
GSH - reduced L-glutathione,
$GS^{\bullet}$ - glutathione thiyl radical,
GSRC - $GS^{\bullet}$ thiyl radical center,
GSSG - glutathione disulfide (oxidized GSH),
MPGly - N-(2-mercapto-propionyl)glycine,
NAC - N-acetylcysteine,
oBQ - 1,2-benzoquinone,
oxoGua - 8-oxo-7-hydroguanine (8-oxoguanine),
ROS - reactive oxygen species,
SQR - semiquinone radical,
SQRC - semiquinone radical center,
T_{m0} - melting-onset temperature.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Highlights

- The antioxidant catechol causes radical-induced DNA damage in the presence of Cu(II).
- Other antioxidants, glutathione, N-acetylcysteine, and N-(2-mercapto-propionyl)glycine, prevent catechol-mediated DNA damage.
- Highest efficiency in DNA-damage prevention is shown by glutathione.
- A novel technique of DNA melting characterization using fluorescent probe DAPI has been developed for assessment of DNA damage.
- The new DNA melting characterization technique can be utilized for the development of new anti-Fenton DNA-protecting drugs.
- The mechanisms leading to different behavior of catechol and GSH derivatives in the interactions with DNA in the presence of Cu(II) have been elucidated.

References

1. Poljsak, B. *Decreasing Oxidative Stress and Retarding the Aging Process*; Nova Science Publishing, Inc.: New York, 2010.
2. Sen, S.; Chakraborty, R.; Sridhar, C.; Reddy, Y. S. R.; De, B. Free radicals, antioxidants, diseases and phytomedicines: Current status and future prospect. *Int. J. Pharm. Sci. Rev. Res.* **2010**, *3*, 91–100.
3. Moussa, S. A. Oxidative stress in *Diabetes mellitus*. *Rom. J. Biophys.* **2008**, *18*, 225–236.
4. Wiernsperger, N. F. Oxidative stress as a therapeutic target in diabetes: Revisiting the controversy. *Diabetes Metab.* **2003**, *29*, 579–585.
5. Markesbery, W. R. Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biol. Med.* **1997**, *23*, 134–147.
6. Perry, G.; Cash, A. D.; Smith, M. A. Alzheimer disease and oxidative stress. *J. Biomed. Biotechnol.* **2002**, *2* (3), 120–123.
7. Yves, C. Oxidative stress and Alzheimer disease[1,2]. *Am. J. Clin. Nutr.* **2000**, *71*, 621S–9S.
8. Krishnan, C. V.; Garnett, M.; Chu, B. Oxidative stress and Parkinson's disease: Electrochemical behavior of hydrogen peroxide in aqueous sodium chloride. *Int. J. Electrochem. Sci.* **2008**, *3*, 1348–1363.
9. Nikam, S.; Nikam, P.; Ahaley, S. K.; Sontakke, A. V. Oxidative stress in Parkinson's disease. *Indian J. Clin. Biochem.* **2009**, *24*, 98–101.
10. Singh, R. P.; Sharad, S.; Kapur, S. Free radicals and oxidative stress in neurodegenerative diseases: Relevance of dietary antioxidants. *J. Indian Acad. Clin. Med.* **2004**, *5*, 218–25.
11. Hamilton, C. A.; H. Miller, W. H.; Al-Benna, S.; Brosnan, M. J.; Drummond, R. D.; McBride, M. W.; Dominiczak, A. F. Strategies to reduce oxidative stress in cardiovascular disease. *Clin. Sci.* **2004**, *106*, 219–234.
12. Lakshmi, S. V. V.; Padmija, G.; Kuppusamy, P.; Kutala, V. K. Oxidative stress in cardiovascular disease. *Indian J. Biochem. Biophys.* **2009**, *46*, 421–440.
13. Madamanchi, N. R.; Vendrov, A.; Runge, M. S. Oxidative stress and vascular disease. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 29–38.
14. Tardif, J.-C. Oxidative stress and coronary heart disease. *Cardiol. Rounds* **2003**, *7*.
15. Galle, J. Oxidative stress in chronic rental failure. *Nephrol., Dial., Transplant.* **2001**, *16*, 2135–2137.
16. Himmelfarb, J.; McMonagle, E.; Freedman, S.; Klenzak, J.; McMenamin, E.; Le, P.; Pupim, L. B.; Ikizler, T. A. PICARD Group: Oxidative stress is increased in critically ill patients with acute renal failure. *J. Am. Soc. Nephrol.* **2004**, *15*, 2449–2456.
17. Halliwell, B. Oxidative stress and cancer: Have we moved forward? *Biochem. J.* **2007**, *401*, 1–11.
18. Tandon, V. R.; Sharma, S.; Mahajan, A.; Bardi, G. H. Oxidative sress : A novel strategy in cancer treatment. *JK Sci.* **2005**, *7*, 1–3.
19. Perluigi, M.; Butterfield, D. A. The identification of protein biomarkers for oxidative stress in Down syndrome. *Expert Rev. Proteomics* **2011**, *8*, 427–429.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

20. Zana, M.; Janka, Z.; Kalman, J. Oxidative stress: A bridge between Down's syndrome and Alzheimer's disease. *Neurobiol. Aging* **2007**, *28*, 648–676.
21. James, S. J.; Cutler, P.; Melnyk, S.; Jernigan, S.; Janak, L.; Gaylor, D. W.; Neubrander, J. A. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *Am. J. Clin. Nutr.* **2004**, *80*, 1611–1617.
22. James, S. J.; Melnyk, S.; Jernigan, S.; Cleves, M. A.; Halsted, C. H.; Wong, D. H.; Cutler, P.; Bock, K.; Boris, M.; Bradstreet, J. J.; Baker, S. M.; Gaylor, D. W. Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *Am. J. Med. Genet., Part B* **2006**, *141B*, 947–956.
23. Masella, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; Giovannini, C. Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *J. Nutr. Biochem.* **2005**, *16*, 577–586.
24. Kohen, R.; Nyska, A. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions and methods for their quantification. *Toxicol. Pathol.* **2002**, *30*, 620–650.
25. Hepel, M.; Stobiecka, M. Detection of Oxidative Stress Biomarkers Using Functional Gold Nanoparticles. In *Fine Particles in Medicine and Pharmacy*; Matijevic, E., Ed.; Springer: New York, 2011; in press.
26. Hepel, M.; Tewksbury, E. Nanogravimetric study of templated copper deposition in ion-channels of self-assembled glutathione films on gold piezoelectrodes. *Electrochim. Acta* **2004**, *49*, 3827–3840.
27. Stobiecka, M.; Coopersmith, K.; Hepel, M. Resonance elastic light scattering (RELS) spectroscopy of fast non-Langmuirian ligand-exchange in glutathione-induced gold nanoparticle assembly. *J. Colloid Interface Sci.* **2010**, *350* (1), 168–177.
28. Mishra, O. P.; Pooniya, V.; Ali, Z.; Upadhyay, R. S.; Prasad, R. Antioxidant status of children with acute renal failure. *Pediatr. Nephrol.* **2008**, *23*, 2047–2051.
29. Baretto, G.; Madureira, D.; Capani, F.; Aon-Bertolino, L.; Saraceno, E.; Alvarez-Giraldez, L. D. The role of catechols and free radicals in benzene toxicity: An oxidative DNA damage pathway. *Environ. Mol. Mutagen.* **2009**, *50*, 771–780.
30. Schweigert, N.; Zehnder, A. J. B.; Eggen, R. I. L. Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.* **2001**, *3*, 81–91.
31. Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J. Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000**, *13*, 135–160.
32. Rice-Evans, C. A.; Diplock, A. T.; Symons, M. C. R. *Techniques in Free Radical Research*; Elsevier: Amsterdam, 1991.
33. Andrews, L. S.; Snyder, R. Toxic Effects of Solvents and Vapors. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*; Klaassen, C. D., Ed.; Mcgraw-Hill, Inc.: New York, 1991.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

34. Eaton, D. L.; Pisabarro, M. T.; Schmidt, K. N.; Vandlen, R.; Chiang, N.; Diehl, L. Interleukin-8 Homologous Polypeptides and Therapeutic Uses Thereof. Patent WO/2006/014505, 2006.

35. Takahashi, N.; Schreiber, J.; Fischer, V.; Mason, R. P. Formation of glutathione-conjugated semiquinones by the reaction of quinones with glutathione: An ESR study. *Arch. Biochem. Biophys. Res. Commun.* **1987**, *252*, 41–48.

36. Segura-Aguilar, J.; Baez, S.; Widersten, M.; Welch, C. J.; Mannervik, B. Human class Mu glutathione transferases, in particular isoenzyme M2-2, catalyze detoxication of the dopamine metabolite aminochromel. *J. Biol. Chem.* **1997**, *272*, 5727–5731.

37. Perron, N. R.; Hodges, J. N.; Jenkins, M.; Brumaghim, J. L. Predicting how polyphenol antioxidants prevent DNA damage by binding to iron. *Inorg. Chem.* **2008**, *47*, 6153–6161.

38. Oikawa, S.; Kawanishi, S. Distinct mechanisms of site-specific DNA damage induced by endogenous reductants in the presence of iron(III) and copper(II). *Biochim. Biophys. Acta* **1998**, *1399*, 19–30.

39. Miura, T.; Muraoka, S.; Fujimoto, Y.; Zhao, K. DNA damage induced by catechol derivatives. *Chem.-Biol. Interact.* **2000**, *126*, 125–136.

40. Nematollahi, D.; Rafiee, M. Electrochemical oxidation of catechols in the presence of acetylacetone. *J. Electroanal. Chem.* **2004**, *566*, 31–37.

41. Bukowska, B.; Kowalska, S. Phenol and catechol induce prehemolytic and hemolytic changes in human erythrocytes. *Toxicol. Lett.* **2004**, *152*, 73–84.

42. Marin, C.; Aguilar, E.; Bonastre, M.; Tolosa, E.; Obeso, J. A. Early administration of entacapone prevents levodopa-induced motor fluctuations in hemiparkinsonian rats. *Exp. Neurol.* **2005**, *192*, 184–193.

43. Ito, N.; Hirose, M.; Imaida, K. Antioxidants: Carcinogenic and Chemopreventive Properties. In *Encyclopedia of Cancer*; Elsevier Science: Amsterdam, 1997; Vol. 1, pp 89−101.

44. Zahid, M.; Gaikwad, N. W.; Rogan, E. G.; Cavalieri, E. L. Inhibition of depurinating estrogen-DNA adduct formation by natural compounds. *Chem. Res. Toxicol.* **2007**, *20*, 1947–1953.

45. Zahid, M.; Saeed, M.; Ali, M. F.; Rogan, E. G.; Cavalieri, E. L. N-acetylcysteine blocks formation of cancer-initiating estrogen-DNA adducts in cells. *Free Radical Biol. Med.* **2010**, *49*, 392–400.

46. Gaikwad, N. W.; Yang, L.; Muti, P.; Meza, J. L.; Pruthi, S.; Ingle, J. N.; Rogan, E. G.; Cavalieri, E. L. *Int. J. Cancer* **2008**, *122*, 1949–1957.

47. Saeed, M.; Zahid, M.; Gunselman, S. J.; Rogan, E.; Cavalieri, E. Slow loss of deoxyribose from N7deoxyguanosine adducts of stradiol-3,4-quinone and hexestrol-3′,4′-quinone. Implications for mutagenic activity. *Steroids* **2005**, *70*, 29–35.

48. Ando, M.; Ueda, K.; Makino, R.; Nishino, Y.; Nishida, H.; Toda, C.; Okamoto, Y.; Kojima, N. Involvement of DNA conformational change induced by rearrangement of copper-coordination geometry in oxidative DNA damages caused by copper and dopamine. *J. Health Sci.* **2009**, *55*, 319–323.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

49. Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C. J.; Telser, J. Roleof oxygen radicals in DNA damage and cancer incidence. *Mol. Cell. Biochem* **2004**, *266*, 37–56.
50. Atkins, P. W.; Friedman, R. S. *Molecular Quantum Mechanics*; Oxford University Press: Oxford, 2004.
51. Hehre, W. J.; Radon, L.; Schleyer, P. R.; Pople, J. A. *Ab-initio Molecular Orbital Theory*; Wiley: New York, 1985.
52. Yamamoto, K.; Kawanishi, S. Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper(II) in and hydrogen peroxide. *J. Biol. Chem.* **1989**, *264*, 15435–15440.
53. Healy, E. F. Quantitative determination of DNA-ligand binding using fluorescence spectroscopy. *J. Chem. Ed.* **2007**, *84*, 1304–1307.
54. Eriksson, S.; Kim, S. K.; Kubista, M.; Norden, B. Binding of 4′,6-diamidino-2-phenylindole (DAPI) to AT regions of DNA: Evidence for an allosteric conformational change. *Biochemistry* **1993**, *32*, 2987–2998.
55. Kim, S. K.; Eriksson, S.; Kubista, M.; Norden, B. Interaction of 4′,6-diamidino-2-phenylindole (DAPI) with poly[d(G-C)$_2$] and poly[d(G-m^{5}C)$_2$]: Evidence for major groove binding of a DNA probe. *J. Am. Chem. Soc.* **1993**, *115*, 3441–3447.
56. Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Strekowski, L.; Boykin, D. W. Binding of 4′,6-diamidino-2-phenylindole (DAPI) to GC and mixed sequences in DNA: Intercalation of a classical groove-binding molecule. *J. Am. Chem. Soc.* **1989**, *111*, 5008–5010.
57. Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Jones, R. L.; Fox, K.; Wydra, R. L.; Strekowski, L., DNA sequence dependent binding modes of 4′,6-diamidino-2-phenylindole (DAPI). *Biochemistry* **1990**, *29*, 8452−8461.
58. Härd, T.; Fan, P.; Kearns, D. R. A fluorescence study of the binding of Hoest 33258 and DAPI to halogenated DNAs. *Photochem. Photobiol.* **1990**, *51*, 77–86.
59. Kubota, Y.; Kubota, K.; Tani, S. DNA binding properties of DAPI (4′,6-diamino-2-phenylindole) analogs having an imidazole ring or a tetrahydropyrimidine ring: Groove-binding and intercalation. *Nucleic Acids Symp. Ser.* **2000**, *44* (1), 53–54.
60. Pineda De Castro, L. F.; Zacharias, M. DAPI binding to the DNA minor groove: A continuum solvent analysis. *J. Mol. Recognit.* **2002**, *15*, 209–220.
61. Banerjee, D.; Pal, S. K. Dynamics in the DNA recognition by DAPI: Exploration of the various binding modes. *J. Phys. Chem. B* **2008**, *112*, 1016–1021.
62. Duguid, J. G.; Bloomfield, V. A.; Benevides, J. M.; Thomas, G. J. DNA melting investigated by differential scanning calorimetry and raman spectroscopy. *Biophy. J.* **1996**, *71*, 3350–3360.
63. Oikawa, S.; Hirosawa, I.; Hirakawa, K.; Kawanishi, S. Site specificity and mechanism of oxidative DNA damage induced by carcinogenic catechol. *Carcinogenesis* **2001**, *22*, 1239–1245.
64. Oikawa, S. Sequence-specific DNA damage by reactive oxygen species: Implications for carcinogenesis and aging. *Environ. Health Prev. Med.* **2005**, *10*, 65–71.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

65. Parcell, S. Sulfur in human nutrition and applicationsin medicine. *Altern. Med. Rev.* **2002**, *7*, 22–44.
66. Sochman, J. N-acetylcysteine in acute cardiology: 10 years later. *J. Am. Coll. Cardiol.* **2002**, *39*, 1422–1428.
67. Zahid, M.; Saeed, M.; Beseler, C.; Rogan, E. G.; Cavalieri, E. L. Resveratrol and N-acetylcysteine block the cancer-initiating step in MCF-10F cells. *Free Radical Biol. Med.* **2011**, *50*, 78–85.
68. Atmaca, G. Antioxidant effects of sulfur-containing amino acids. *Yonsei Med. J.* **2004**, *45*, 776–788.
69. Wang, C.; Zhao, J.; Zhang, D.; Z., Y. Detection of DNA damage induced by hydroquinone and catechol using an electrochemical DNA biosensor. *Aust. J. Chem.* **2009**, *62*, 1181–1184.
70. Brett, A. M. O.; Diculescu, V. C. Electrochemical study of quercetin-DNA interactions. Part II. In situ sensing with DNA biosensors. *Biochemistry* **2004**, *64*, 143–150.
71. Brett, A. M. O.; Piedade, J. A. P.; Serrano, S. H. P. Electrochemical oxidation of 8-oxoguanine. *Electroanalysis* **2000**, *12*, 969–972.
72. Ye, Y.; Munk, B. H.; Muller, J. G.; Cogbill, A.; Burrows, C. J.; Schlegel, H. B. Mechanistic and kinetic aspects of the formation of guanidinohydantoin and spiro iminohydantoin under acid conditions. *Chem. Res. Toxicol.* **2009**, *22*, 526–535.
73. Hosford, M. E.; Muller, J. G.; Burrows, C. J. Spermine participates in oxidative damage of guanosine and 8-oxoguanosine leading to deoxyribosylurea formation. *J. Am. Chem. Soc.* **2004**, *126*, 9540–9541.
74. Schweigert, N.; Acero, J. L.; von Gunten, U.; Canonica, S.; Zehnder, A. J. B.; Eggen, R. I. L. DNA degradation by mixture of copper and catechol is caused by DNA-copper-hydroperoxo complex, probably DNA-Cu(I)OOH. *Environ. Mol. Mutagen.* **2000**, *36*, 5–12.
75. Osterberg, R.; Ligaarden, R.; Persson, D. Copper(I) complexes with penicillamine and glutathione. *J. Inorg. Biochem.* **1979**, *10*, 341–355.
76. Ramoutar, R.; Brumaghim, J. L. Investigating the antioxidant properties of oxo-sulfur compounds on metal-mediated DNA damage. *Main Group Chem.* **2007**, *6*, 143–153.
77. Carrasco-Pozo, C.; Aliaga, M. E.; Olea-Azar, C.; Speisky, H. Double edge redox-implications for the interaction between endogeneous thiols and copper ions: In vitro studies. *Bioorg. Med. Chem.* **2008**, *16*, 9795–9803.
78. Hsu-Kim, H. Stability of metal-glutathione complexes during oxidation by hydrogen peroxide and Cu(II)-catalysis. *Environ. Sci. Technol.* **2007**, *41*, 2338–2342.
79. Battin, E. E.; Brumaghim, J. L. Metal specificity in DNA damage prevention by sulfur antioxidants. *J. Inorg. Biochem.* **2008**, *102*, 2036–2042.
80. Roots, R.; Okada, S. Estimation of life times and diffusion distances of radicals involved in X-ray-induced DNA strand breaks or killing of mammalian cells. *Radiat. Res.* **1975**, *64*, 306–320.
81. Baxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. Diffusion coefficient for OH radical. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

82. Schafer, F. Q.; Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biol. Med.* **2001**, *30*, 1191–1212.
83. Hiroi, M.; Ogihara, T.; Hirano, K.; Hasegawa, M.; Morinobu, T.; Tamai, H.; Niki, E. Regulation of apoptosis by glutathione redox state in PC12 cells exposed simultaneously to iron and ascorbic acid. *Free Radical Biol. Med.* **2005**, *38*, 1057–1072.
84. Battin, E. E.; Perron, N. R.; Brumaghim, J. L. The central role of metal coordination in selenium antioxidant activity. *Inorg. Chem.* **2006**, *45*, 499–501.
85. Balla, J.; Kiss, T.; Jameson, R. F. Copper(II)-catalyzed oxidation of catechol by molecular oxygen in aqueous solution. *Inorg. Chem.* **1992**, *31*, 58–62.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch006

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

Chapter 7

Antioxidant Effectiveness in Preventing Paraquat-Mediated Oxidative DNA Damage in the Presence of H_2O_2

Magdalena Stobiecka, Amanda Prance, Kaitlin Coopersmith, and Maria Hepel*

Department of Chemistry, State University of New York at Potsdam, Potsdam, NY 136756
***E-mail: hepelmr@potsdam.edu**

Many biological processes, including aging, mutagenesis, and carcinogenesis, are induced by DNA damage inflicted under oxidative stress conditions. Surprisingly, many organic antioxidants that can neutralize free radicals and reactive oxygen species (ROS), can also act under certain conditions as pro-oxidants and be a source of DNA-damaging ROS. One such compound, paraquat (PQ), which is a commonly used herbicide, has been found to generate ROS and cause serious DNA damage, including base oxidation and strand breaks. In this work, we have investigated the extent of PQ-induced DNA damage occurring in the presence of H_2O_2 which by itself in absence of any catalysts does not damage the DNA. The DNA damage was assessed using the electrochemical biosensor method with immobilized-DNA sensing film electrodes. The DNA damage was also evaluated using DNA-melting charateristics recorded using a novel DAPI fluorescent probe able to interact with the DNA double helix. Theses techniques have also been applied in measurements of DNA-protective abilities of antioxidants: melatonin and L-carnitine. These compounds are able to penetrate the brain barrier and thus are important for treatment of oxidative stress in brain which is inaccessible to other antioxidants. The oxidative stress causes devastation in brain in diseases, such as the Down syndrome and autism, and is also affecting dialysis patients and others.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

We have found that both melatonin and L-carnitine are able to attenuate the PQ-mediated DNA damage. The results of this investigation indicate that the DNA-protecting efficiency of melatonin is higher than that of L-carnitine with critical concentrations of these antioxidants equal to 30 μM and 250 μM, respectively, for a solution of 100 μM PQ + 167 μM H_2O_2.

Keywords: Paraquat; Oxidative DNA damage; Melatonin antioxidant; Reactive oxygen species (ROS); DNA melting determination; DAPI fluorescent probe; L-carnitine antioxidant; Nile Blue redox probe

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

Introduction

Oxidative damage to DNA double-helix plays a critical part in many biological processes, such as aging, mutagenesis, and carcinogenesis (*1*, *2*). A range of pesticides and herbicides can cause a DNA damage (*3*, *4*). In this work, we have investigated the extent of DNA damage done by herbicide paraquat (PQ) in the presence of H_2O_2 using electrochemical biosensor method and fluorescence techniques. The assessment of DNA-protection against PQ-mediated DNA damage has been performed utilizing antioxidants melatonin (*5*), (*6*) and L-carnitine (*7–10*). These antioxidants represent a group of few compounds able to penetrate the brain barrier and thus offering unique property which can be exploited for fighting the oxidative stress in brain tissue. This is important in diseases such as the Down syndrome, autism, and others. The level of oxidative DNA damage in brain is at least 10-fold higher than in nuclear DNA (*11*, *12*).

Paraquat (methyl viologen) is a widely utilized, ubiquitous herbicide which is nonselective and can cause fatal intoxication in humans and animals (*13*). It is a toxin known to target the dopaminergic neurons and could induce neurodegenerative diseases (*14–16*). Paraquat causes extensive damage to lungs, kidneys, brain, and liver and ulceration of the digestive tract (*6*, *17*). Paraquat is a well-known environmental risk factor for Parkinson's disease (*18*, *19*). Its action is associated with the low redox potentials of paraquat couples: $PQ^{2+}/PQ^{\bullet+}$ and $PQ^{\bullet+}/PQ^0$ (or using methyl viologen nomenclature: $MV^{2+}/MV^{\bullet+}$ and $MV^{\bullet+}/MV^0$). Therefore, PQ can react readily with molecular oxygen as an electron donor and form a paraquat radical. This radical is also formed by the interaction of PQ with the mixed-function system of microsomes in the presence of NADPH. The superoxide anions (O_2^-) are formed in this process under aerobic conditions (*20*, *21*). The investigations clearly indicate that paraquat inflicts damage to calf thymus DNA when incubated with mice brain, lung homogenate and liver microsomes. The DNA damage can be diminished by adding scavengers of $^\bullet OH$ radicals which are formed during PQ-mediated DNA-damage and are the main cause of damage.

The reactive oxygen species at high concentrations are toxic for living cells, damaging all major cellular constituents but under the conditions of low, physiologically-relevant concentrations, they can also regulate basic biochemical

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

mechanisms linked to vital cellular functionalities (*22*). Paraquat has been found to initiate the formation of reactive oxygen species (ROS), such as $HO^\bullet$, H_2O_2, $O_2^{\bullet-}$, and to induce the DNA damage. Yamamoto and Mohanan have shown that the incubation of brain or lung homogenates with PQ and calf thymus DNA for 90 min, leads to a PQ-mediated DNA damage that progressed in a concentration-dependent manner (*21*). Paraquat can induce dopaminergic cell death and inflict damage to dopaminergic neurons in the nigrostriatum. The latter is related to oxidative stress caused by excessive ROS production (*23–25*). Ross et al. have reported that mouse lymphoblasts in a culture showed an increased loss of colony-forming ability after an exposure to PQ. This has been attributed to the DNA damage occurring via single strand breaks. It was explored using the alkaline elution technique (*26*). Ali and coworkers in their investigations have indicated that the PQ-generated ROS, under aerobic conditions in vitro, caused oxidation of guanine to 8-oxoguanine and led to a modification of the Bam H1 restriction site on pBR322 DNA (*17*). Tokunaga et al. have reported that paraquat increased markedly the 8-hydroxy-deoxyguanosine contents in various organs, particularly in brain, lung and heart, showing a clear involvement of PQ in oxidative DNA damage (*27*). In general, guanine of GC base-pairs in DNA is the most prone to oxidation among all nitrogen bases (*3*, *28*, *29*). In reported cases of PQ-induced acute renal failure, the administration of antioxidants α-tocoferol, dimethylthiourea or deferoxamine, has been advised (*20*). Chagas et al. have studied the penetration of paraquat through the leaf cuticule. It has been found that PQ quickly decreases the leaf photosynthetic activity by destroying membranes within hours. Necrotic lesions are formed on the leaf surface in the advanced stages of phototoxicity (*30*).

The protective action of melatonin against oxidative DNA damage has been studied by Sliwinski et al. (*31*). Melatonin possesses oncostatic and anti-cancer properties and is one the few compounds that can cross the brain barrier and extend its protective ability in the brain. The mechanism underlying the anti-cancer properties involves several processes, including inhibition of the uptake of fatty-acid growth–factor by the tumor tissue, inhibition of telomerase activity in tumors and a reduction of endothelin-1 synthesis in cancer cells. According to Melchiori et al. (*5*), melatonin reduces PQ-induced genotoxicity in mice. Since the DNA damage and repair play the key parts in cancer cell growth and proliferation, the anticancer properties of melatonin are so important. Several types of mutations are involved in cell transformation that arise from DNA damage by ROS and cause base modifications. Melatonin interacts with H_2O_2 producing its direct metabolite N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) which is also active and shows antioxidant properties. Up to a concentration of at least 1000 μM, melatonin does not induce DNA damage in human lymphocytes. Melatonin and AFMK have been found to decrease significantly the tail in comet essay of DNA damaged by ROS in cells exposed to H_2O_2 at all tested concentrations. Melatonin and AFMK exhibit strong antioxidant properties (*31*). The results of melatonin investigations indicate that melatonin increases considerably the kinetics of DNA repair, diminishing H_2O_2-induced DNA damage to the level of control cell after 10 min, while cells incubated in the absence of melatonin required 120 min to complete the repair of their DNA. It has been shown that melatonin inactivates hydrogen peroxide and that this effect could be, at least in a significant part, responsible for its protective action. Therefore, the beneficial effect of melatonin on DNA damage may have at least two aspects: (i) preventing its induction and (ii) repairing it. It has been shown that the former is achieved by a chemical inactivation of hydrogen peroxide whereas the latter aspect needs further studies.

Ross and Krieger have found that spermine is the most potent inhibitor of PQ uptake in rat lung slides. This finding is in agreement with the reported high potency of α,ω-diaminoalkanes with nitrogen atoms separated by four or seven carbons. The structural requirements for a compound to effectively occupy or block this receptor are: (1) two or more positively charged nitrogens; (2) maximum of the positive charge surrounding the nitrogens; (3) an apolar group increasing the compound solubility; and (4) minimal steric hindrance near the nitrogen atoms (*32*). While melatonin conforms to some of the conditions listed above, it lacks flexibility and for this reason may not function in the same way as the α,ω-diaminoalkanes do.

L-carnitine, a mitochondrial carrier of fatty acids, has been found to inhibit lipid peroxidation (*7*, *8*, *33*), propionic acid induced DNA-damage in propionic acidemia (*8*), acrylamide-induced DNA damage (*34*). inhibit accumulation of age-related oxidative DNA damage (*12*), and protect mammalian cells against chromosome aberration (*10*). According to Berni et al. (*9*), L-carnitine enhances the rate and extent of the DNA repair. Cells treated with L-carnitine have shown a reduction of all types of chromosomal aberrations caused by oxidative stress. It has been reported that L-carnitine protects myocardium against ischemia (*35*), heart failure (*36*), and myocardial infarction (*37*). L-carnitine has also a neuroprotective role in the brain (*38*). Several groups have suggested that L-carnitine protects cells by scavenging radicals (*39*) and increasing the rate of single-strand break repair (*40*, *41*). At very high concentration (50 mM), L-carnitine reverses its action, synergistically increasing the PQ-toxicity and increasing the depletion of intracellular glutathione.

In this work, we have investigated the extent of DNA damage induced by paraquat in the presence of H_2O_2 using the electrochemical biosensor intercalation method (*42–44*) with a redox probe Nile Blue (NB) and a fluorescence quenching method. The DNA damage was assessed from the DNA melting characteristics and the NB-probe intercalation capacity which we have developed recently for the evaluation of atrazine effect on DNA conformation (*43*). These methods were also used for the assessment of the DNA-protection propensity of melatonin and L-carnitine. The differences in effectiveness of DNA protection by these two antioxidants have been determined and the mechanism of action has been elucidated.

2. Materials and Methods

2.1. Chemicals

Methyl viologen (paraquat, PQ), melatonin, deoxyribonucleic acid from calf thymus, sodium salt (ctDNA), mercaptopropionic acid (MPA),

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), redox active dye Nile Blue A (NB), Trizma hydrochloride (TRIS-HCl) and fluorescent dye 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.). L(-)-Carnitine was obtained from Acros Organics (New Jersey, U.S.A.). Sodium phosphate monobasic dehydrate, sodium chloride, potassium chloride and sodium phosphate dibasic were obtained from Fisher Scientific Company (Pittsburgh, PA, U.S.A.). The dsDNA stock solution (1 mg/mL) was prepared at least 24 h before measurements. The short synthetic oligonucleotides were purchased from Eurofins MWG/Operon (Huntsville, AL, U.S.A.). The cyclic voltammetry measurements were performed in the phosphate buffer saline solution (PBS), which consists of 0.15 M NaCl, 2 mM KCl, and 0.02 M phosphate buffer pH = 7.4. In fluorimetric measurements TRIS-HCl buffer (0.02 M, pH 7.5) was used. Solutions were prepared using Millipore (Billerica, MA, U.S.A.) Milli-Q deionized water (conductivity $\sigma = 55$ nS/cm).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

2.2. Preparation of DNA Biosensors on the Gold Electrodes

10 mM mercaptopropionic acid (MPA) was immobilized onto clean gold electrode for 1 h. After washing in water and PBS buffer, the oligonucleotide probe ((5′⇒3′) $NH_2C_6H_{12}$-ATTCGACAGGGATAGTTCGA) was attached via amide bonds to self assembly monolayer of MPA. Fresh prepared N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) solution was added to 0.02 M PBS buffer for activation of carboxyl group and then NH_2-modified oligonucleotide solution (100 μM) was injected for 1h. The final concentration of NH_2-oligonucleotides and EDC were 1 μM and 0.1 M, respectively. The hybridization process was performed by injecting complementary oligonucleotide (100 μM solution, (5′⇒3′) TCGAACTATCCCTGTCGAAT) to PBS for 1h. Final concentration of the complementary oligonucleotides was 1μM. Such prepared biosensor was rinsed with 0.02 M PBS buffer and used for testing DNA damage by paraquat (PQ).

2.3. Electrochemical Measurements

For cyclic voltammetric measurements the standard electrochemical setup - Potentiostat/Galvanostat Model PS-205B with a three-electrode configuration from Elchema (Potsdam, NY, U.S.A.) with a Data Logger and Control System, Model DAQ-716v, operating under Voltscan 5.0 data acquisition and processing software was employed. The double-junction saturated Ag/AgCl electrode was used as the reference electrode and Pt wire as the counter electrode. The gold electrodes (Au) with an area of 1 mm^2, obtained from Elchema (Potsdam, NY, U.S.A.) were used as working electrode. All cyclic voltammetry (CV) measurements were performed in 0.02 M PBS buffer, pH 7.4 with the potential window from 0 V to -0.6V, and a scan rate 100 mV/s. The surfaces of AuE electrodes were first polished with wet 0.3 and 0.05 μm alumina slurry (Coating Service Department, Indianapolis, U.S.A.) on a flat pad and rinsed repeatedly with water to remove any alumina residue. The polished electrodes were then cleaned electrochemically in the 1M KOH and 0.1 M H_2SO_4 solution. The solutions were deoxygenated by purging with argon for 15 min, and the potential was cycled between -400 and -1800 mV or between 0 and 1500 mV (versus a Ag/AgCl reference electrode) with a scan rate of 100 mV s-1 until the cyclic voltammograms showed no further change, respectively for NaOH and H_2SO_4 solution.

2.4. Fluorimetric Measurements

The fluorimetric measurements were recorded using LS55 Spectrometer (Perkin Elmer, Waltham, MA, U.S.A.) equipped with 20 kW Xenon light source operating in 8 μs pulsing mode. Pulse width at half height was less than 10 μs. Separate monochromators for the incident beam and the detector beam enabled to use monochromatic radiation with wavelengths from 200 nm to 800 nm with 1 nm resolution. The dual detector system consisted of a photomultiplier tube (PMT) and an avalanche photodiode. Both the excitation and emission slit widths were set to 5.0 nm and scan speed 500 nm/min. Measurements were performed in TRIS-HCl buffer (0.02 M, pH 7.5).

2.5. Molecular Dynamic Simulations and Quantum Mechanical Calculations

Quantum mechanical calculations of electronic structures for an interaction between a model DNA molecule, paraquat (PQ), melatonin (Mel) antioxidant and hydrogen peroxide were performed using modified Hartree-Fock methods with 6-31G* basis set and pseudopotentials, semi-empirical PM3 method, and density functional theory (DFT) with B3LYP functional. The molecular dynamics simulations and quantum mechanical calculations were carried out using procedures embedded in Wavefunction (Irvine, CA, U.S.A.) Spartan 6. The electron density and local density of states are expressed in atomic units, au^{-3}, where 1 au = 0.52916 Å and 1 au^{-3} = 6.7491 $Å^{-3}$.

3. Results and Discussion

3.1. Detection of DNA Damage Using Electrochemical Biosensors

The investigation of the DNA damage caused by paraquat was performed using DNA hybridization sensing with electrochemical transduction (*42–44*) and fluorimetric measurements.

Figure 1 shows the formulas of analytes and probes used in the experiments, including: fluorescent probe 4′,6-diamidino-2-phenylindole (DAPI), melatonin (Mel), paraquat (PQ, methyl viologen), L-carnitine, and redox probe Nile Blue (NB). The redox active dye Nile Blue (NB) was used in cyclic voltammetric and fluorimetric measurements. The fluorescence dye 4′,6 diamidino-2-phenylindole (DAPI) was used for checking the melting-onset temperature (T_{mo}) of ctDNA before and after the damage induced by herbicide paraquat (PQ) in the presence of H_2O_2. The melatonin (Mel) and L-carnitine antioxidants were used to prevent the DNA damaged.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

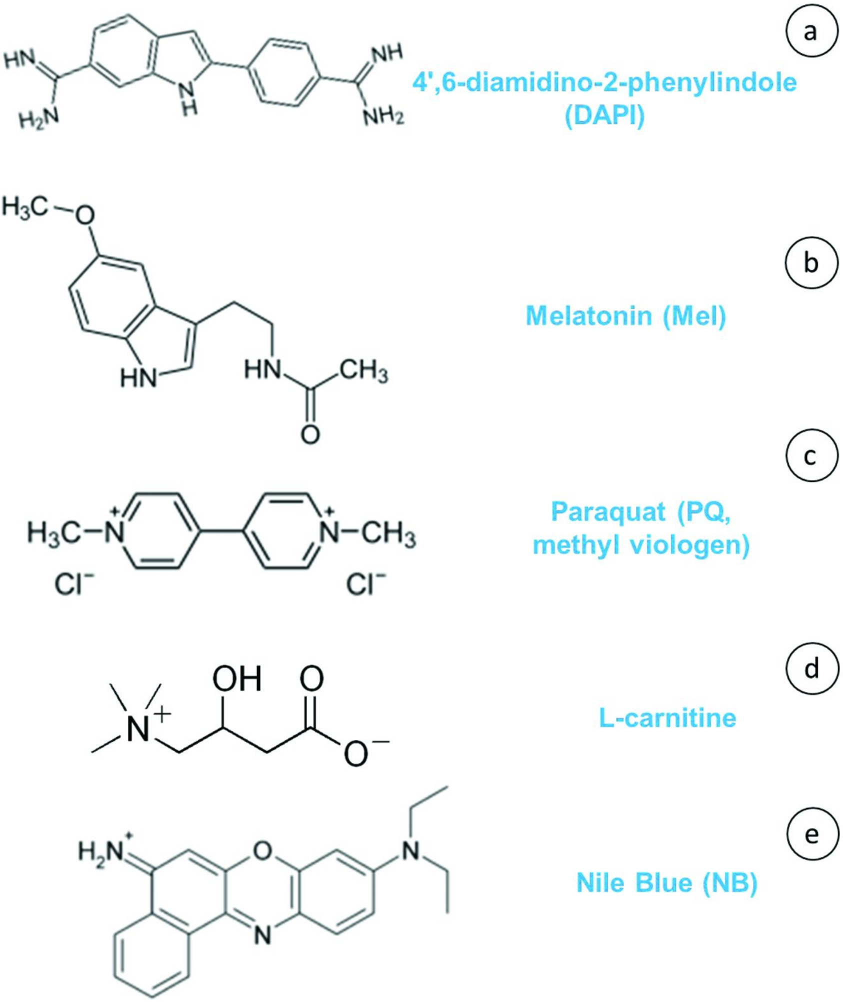

a
4',6-diamidino-2-phenylindole (DAPI)
b
Melatonin (Mel)
c
Paraquat (PQ, methyl viologen)
d
L-carnitine
e
Nile Blue (NB)

Figure 1. The analytes and probes used in experiments: a) 4',6-diamidino-2-phenylindole (DAPI), (b) Melatonin (Mel), (c) Paraquat (PQ, methyl viologen), (d) L-carnitine, (e) Nile Blue (NB).

In Figure 2, the scheme of a DNA biosensor film immobilized on a Au electrode is presented. First, the carboxylic groups of mercaptopropionic acid (MPA), that formed a self-assembled monolayer (SAM) on a gold electrode were activated by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) solution to enable the immobilization of NH_2-modified oligonucleotides. Next, the hybridization with complementary oligonucleotides was performed. The DNA biosensor was then used for testing of interactions of DNA with paraquat (PQ) using a redox dye NB as the probe intercalator and marker of DNA damage.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

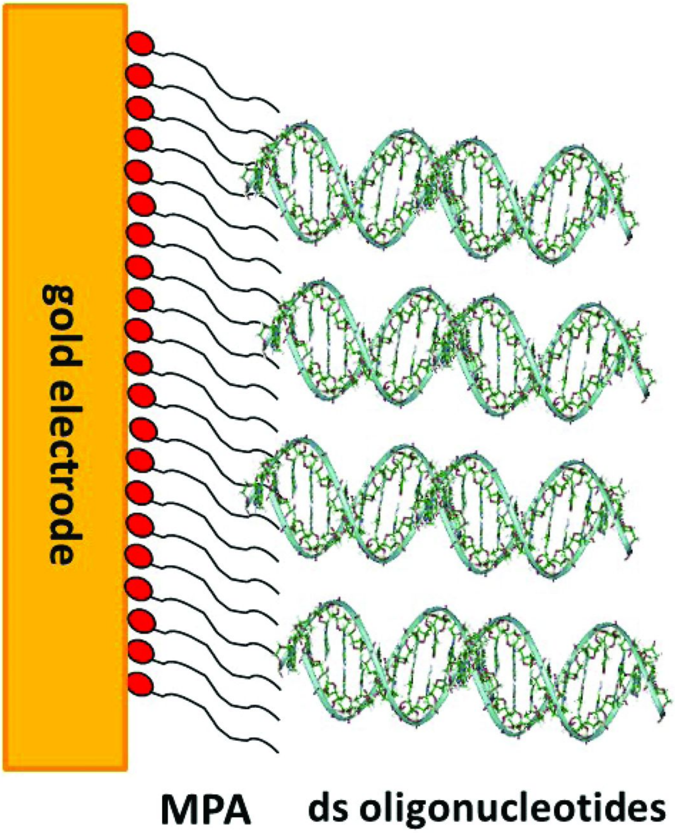


Figure 2. Scheme of the modification of the gold electrode.

The cyclic voltammograms were recorded in the 0.02 M PBS buffer, pH = 7.4 after soaking the biosensors in a 100 µM NB solution for 10 min. The cathodic peak currents (I_{pc}) of the reduction process of NB intercalated into DNA were utilized as a measure of the NB uptake by a film. The DNA damage by PQ was evaluated by immersing the biosensor in a 100 µM of herbicide solution for 10, 35, 55 or 80 min. and then once again incubating in NB solution and checking in PBS buffer. The changes in the redox peak current of the intercalator probe NB before and after interactions of DNA with herbicide were quantified and indicated the damage of DNA. In Figure 3 and Figure 4, curve 1 depicts the current value of biosensor before interactions with redox electroactive NB marker (I_b).

After soaking the biosensor for 10 min in 100 µM NB solution, well defined cathodic peak appeared in PBS buffer (I_{pc0}). It indicates on the intercalation of NB into DNA duplex (Figures 3 and 4, curve 2). After interaction of DNA with PQ the I_{pcPQ} of a NB intercalated to DNA was diminished (Figures 3 and 4, curve 3). After once again soaking of the biosensor for 10 min in 100 µM NB solution, the change of the redox current of NB dye (curve 4) in comparison to that before the interaction with herbicide (curve 2) was observed. The DNA damage φ was measured as the relative current increase in the NB uptake after interaction of DNA with herbicide:

$$\varphi = 100\ (I_{\mathrm{pcPQ}} - I_{\mathrm{b1}})/(I_{\mathrm{pc0}} - I_{\mathrm{b0}}) \qquad (1)$$

where $I_{\mathrm{pc,0}}$ and $I_{\mathrm{pc,PQ}}$ are the cathodic peak currents for the reduction of NB intercalated in dsDNA helix on electrode before and after interactions of DNA with PQ, respectively; I_{b0} and I_{b1} are the background currents. During the first 60 min, the cathodic current of NB intercalated to dsDNA after interactions with PQ increased about 9.8 - 19.6 % in comparison with current of NB intercalated into undamaged DNA. It indicates on the expanding structure of DNA due to interactions of herbicide with a dsDNA helix leading to the increase of uptake of Nile Blue molecules between base pairs and the increase of redox signal of NB in comparison to a undamaged dsDNA was observed. Similar results were obtained after interaction of herbicide atrazine with DNA (*4*). The uptake of NB in DNA after interactions with Atz for 40 min was φ = 44 %. The damage of DNA by atrazine caused unwinding of double strands of the helix and an increase of the NB uptake.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

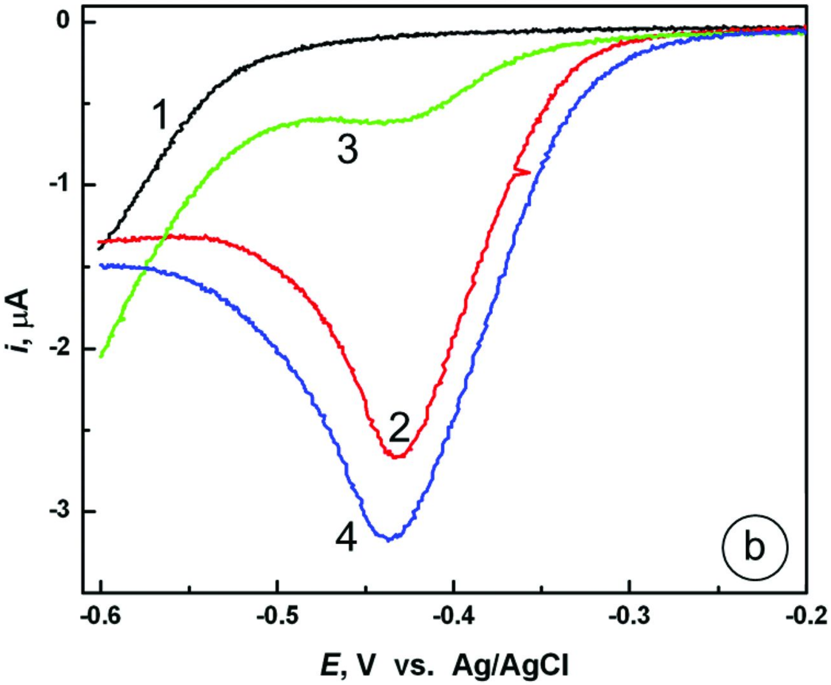


Figure 3. Linear potential scan voltammetry for a QC/Au/MPA-dsDNA$_{20bp}$ electrode in 0.02 M PBS (1), pH=7.4, after subsequent treatments: (2) 10 min soaking in NB solution (100 µM); (3) after 35 min soaking in PQ solution (100 µM); (4) after soaking in PQ and 10 min soaking in NB solution (100 µM); scan rate v = 100 mV/s.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

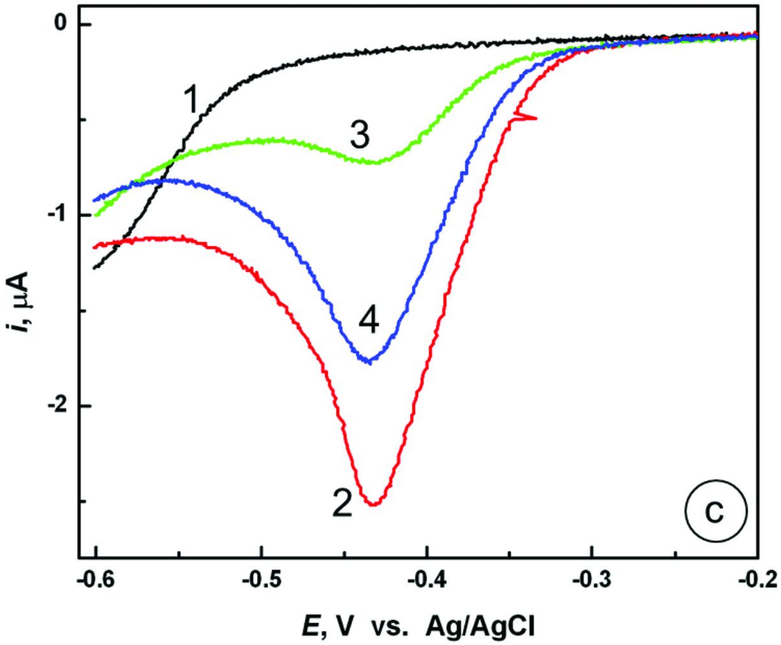


Figure 4. Linear potential scan voltammetry for a QC/Au/MPA-dsDNA$_{20bp}$ electrode in 0.02 M PBS (1), pH=7.4, after subsequent treatments: (2) 10 min soaking in NB solution (100 µM); (3) after 80 min soaking in PQ solution (100 µM); (4) after soaking in PQ and 10 min soaking in NB solution (100 µM); scan rate v = 100 mV/s.

It is known, that PQ reversibly binds to DNA by ionic interactions with negatively charged phosphate residues of DNA and can also intercalate into DNA duplex. Minchin (*45*) has shown that paraquat binds to calf thymus DNA stabilizing the double helix and competitively inhibited ethidium bromide binding to DNA. Plausible explanation of the paraquat action is based on DNA conformational changes that involve lengthening and unwinding of DNA molecules. Such conformational transitions are also observed for other intercalators and are consistent with DNA behavior in interactions with herbicide atrazine (*4*, *42*).

After 60 min of interactions of the oligonucleotides with PQ, the decrease of current of the NB intercalated to DNA was observed. It indicates on damage of DNA evoked by strand breaks and DNA fragmentation. PQ caused first unwinding of the dsDNA helix and then cleaving, therefore less of NB could intercalate into a DNA duplex and the cathodic current signal was decreased about 32.1% (Figure 5).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

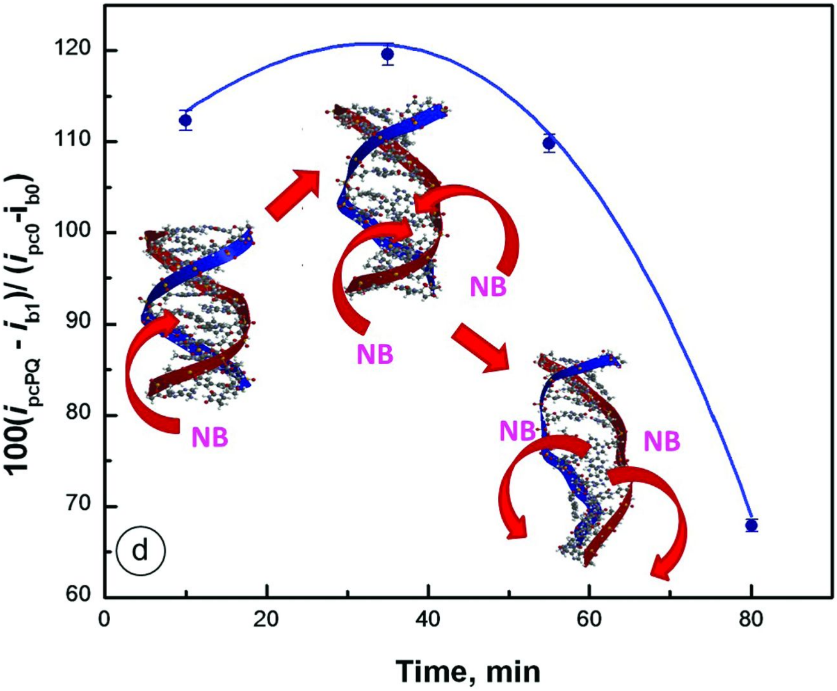


Figure 5. Dependence of NB current after DNA damage on time of interactions of oligonucleotides with PQ.

3.2. Detection of DNA Damage Using Fluorimetric Measurements

To confirm results obtained for the oligonucleotides immobilized on gold electrodes, separate experiments with ctDNA in 20 mM phosphate buffer, pH 7.4 were performed. The fluorimetric method was used for evaluation of DNA damage by PQ. In Figure 6, the emission spectra of a fluorescence dye NB centered at $\lambda = 672$ nm are presented before and after intercalation of the dye into the undamaged and damaged DNA duplex. NB dye become fluorescence inactive after the intercalation into the dsDNA and fluorescence signal for NB decreased from $I = 737$ to $I = 647$ a.u., respectively (Figure 6, curve 1 and curve 2).

Also, a stronger quenching of fluorescence signal of NB was observed after the interaction of dsDNA with PQ for 1 and 60 min than with a more rigid undamaged DNA double helix structure (Figure 6, curves 3 and 4). It indicates that more NB was intercalated into DNA helix. The increase in the intercalation capacity for DNA to fluorescence dye is clearly associated with damaging interaction of herbicide with the nucleic acid. The PQ intercalation caused underwinding of dsDNA and lengthening of DNA molecule through the increase of the inter-base spacing in the base stacks leading to the increased uptake of NB probe. Hence, the fluorescently active NB molecules were deactivated due to the higher capacity of damaged DNA for NB molecules. Minchin (*45*) also reported that intercalation of

PQ caused lengthening of the DNA molecule and competitive inhibition of binding to DNA for another intercalator dye ethidium bromide.

The fluorescence spectrum recorded after 195 min of interactions of DNA with PQ shows opposite dependence, an increase of emission signal of NB (Figure 6, curve 5). This result was caused by DNA damage. Due to the unwinding of double stranded DNA, single strand breaking and DNA cleaving, the NB dye is released from DNA helix and becomes fluorescence active. The intensity signal of the dye in the solution after long time of interaction of the DNA with PQ increased from $I = 566$ to $I = 687$ a.u. The data obtained in fluorimetric measurements are consistent with findings obtained in cyclic voltammetric measurements.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

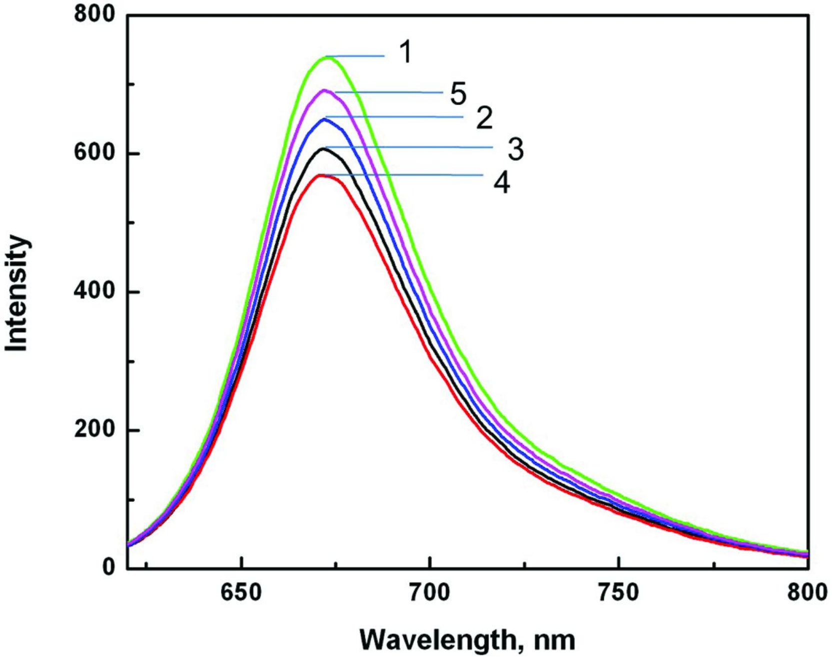


Figure 6. Fluorescence emission spectra for: (1) NB alone, (2) NB intercalated to ctDNA before interactions of DNA with PQ and (3-5) same as (2) but after interactions of DNA with PQ, interaction time: (3) t_{inc} = 1 min; (4) t_{inc} = 60 min; (5) t_{inc} = 195 min; phosphate buffer (0.02 M, pH 7.4), C_{NB} =1 µM, C_{ctDNA} =1.45 µM bp, C_{PQ} =100 µM.

3.3. Detection of DNA Damage by Measuring Changes in DNA Melting-Onset Temperature Using DAPI Fluorescent Dye

The melting-onset temperature (T_{mo}) of the ctDNA duplex was assessed during the fluorescence measurements and was used as a DNA damage indicator (Figures 7-9). The investigations were carried out using fluorescence dye 4′,6 diamidino-2-phenylindole (DAPI) which binds with DNA in the minor groove

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

mainly with AT base-pairs and intercalates at GC or mixed AT and GC DNA sequences. DAPI shows enhancement of the emission signal centered at λ = 450 nm after interactions with double stranded DNA (*46–51*). Figure 7 shows the melting curves obtained after heating of a 166.7 nM DAPI dye with 2.023 μM bp ctDNA solution before and after damage done by PQ alone or PQ in the presence of H_2O_2 (curve 1, 2 and 3, respectively). The melting-onset temperature for undamaged DNA was determined from the melting curve as 77.6 °C. Exposure of DNA to 100 μM paraquat resulted in a slightly decreased T_{mo} indicating that under these conditions rather insignificant DNA damage occurred. The DNA damage was enhanced after the addition of 166.7 μM H_2O_2 to 100 μM PQ solution and 2.023 μM bp ctDNA and therefore, a bigger shift (T_{mo} = 71.1 °C) towards the lower temperature was detected. The hydrogen peroxide can cause base alterations, modification, and strands break (*22*).

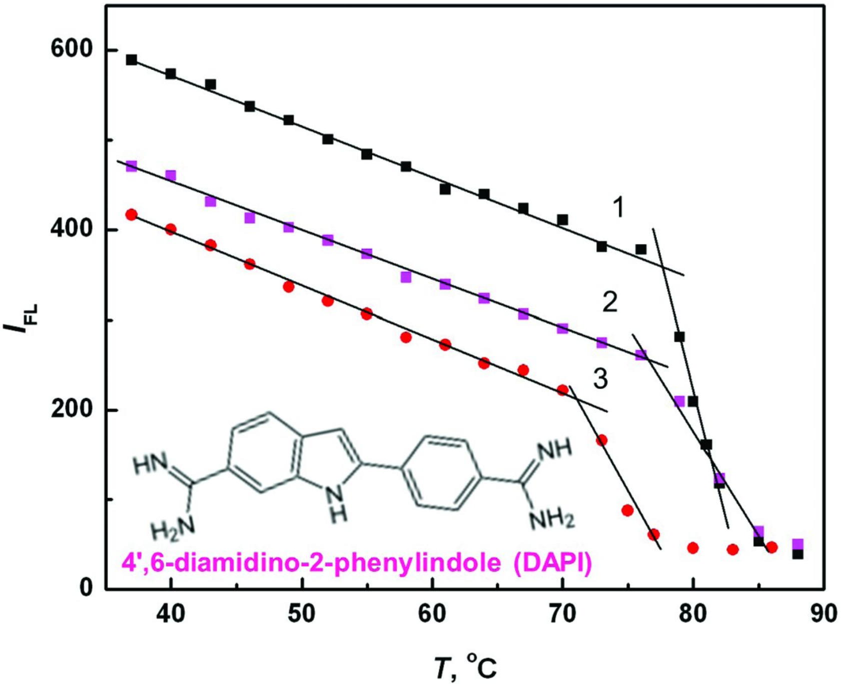


Figure 7. Dependence of DAPI-probe fluorescence on temperature after interactions of ctDNA alone (1), with paraquat (2) and paraquat + H_2O_2 (3), in TRIS-HCl buffer (20 mM, pH = 7.5); C_{ctDNA} = 2.023 μM bp; C_{DAPI} = 166.7 nM; C_{PQ} = 100 μM; C_{H2O2}= 166.7 μM; t_{inc} = 1 h in 37 °C.

Figure 8 depicts the dependence of DAPI fluorescence signal on temperature after interactions of DNA with 166.7 μM H_2O_2 and different concentration of paraquat. Surprisingly, it appears that the highest concentration of PQ used in this experiment did not cause the largest T_{mo} decrease. It is most likely due to the ability of the herbicide to reversibly cross-link DNA molecules and stabilize the helix, similar to the effect observed for polyamines. It is known, that the endogenous amines, such as putrescine and spermidine, after binding with phosphate groups of the nucleic acid, enhance the T_{mo} of DNA. The similarity between these molecules is related to the spatial separation of the nitrogen group on each molecule (*32*), (*45*). In putrescine, spermidine and spermine, two of the nitrogen moieties are separated by a four-methylene group bridge, a distance of approx. 6.6 Å. The pyridyl nitrogens in the paraquat molecule are approx. 7 Å apart (*45*).

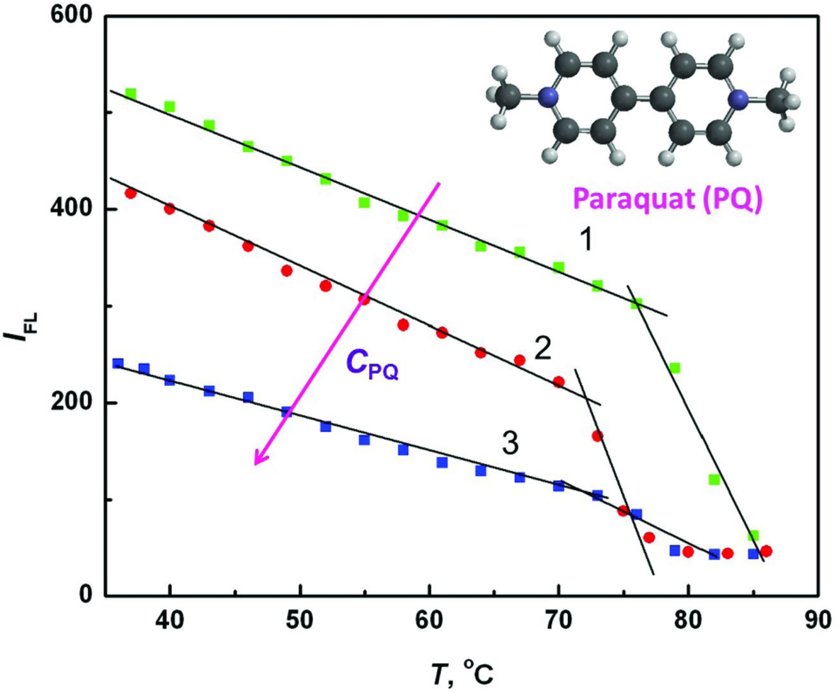


Figure 8. Dependence of DAPI-probe fluorescence on temperature after interactions of 2.023 μM bp ctDNA with different concentrations of the paraquat and 166.7 μM H_2O_2 . C_{PQ} [μM]: (1) 50 (2) 100, (3) 500 in TRIS-HCl buffer (20 mM, pH = 7.5); C_{ctDNA} = 2.023 μM bp; C_{DAPI} = 166.7 nM; t_{inc} = 1 h in 37 °C.

Most likely, what we observe here are two kinds of actions of the PQ. Firstly, PQ causes a DNA damage by underwinding, followed by DNA cleaving. Secondly, PQ is cross-linking DNA molecules. The highest concentration of PQ caused the most DNA damage associated with underwinding and strand breaking, leading to the lowest intensity of DAPI fluorescence emission. The DAPI molecules do not match precisely the damaged DNA, therefore, the fluorescence signal of the dye is lower in comparison to that for the undamaged double helix. On the other hand, high concentration of PQ molecules caused the stabilization of DNA helix and increase of T_{mo}.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

3.4. Effect of Melatonin and L-Carnitine Antioxidants on PQ-Induced DNA Damage

Antioxidative effects of melatonin and L-carnitine were examined by measuring the inhibition of DNA damage correlated with the decrease of the melting-onset temperature T_{mo} of ctDNA. The comparison of T_{mo} vs. C characteristics for melatonin (curve 1) and L-carnitine (curve 2) on PQ-mediated DNA damage is shown in Figure 9. DNA was subjected to the damage induced by 100 µM PQ in the presence of 166.7 µM H_2O_2. The inset in Figure 9 presents the temperature dependence of DAPI fluorescence emission for several concentrations of melatonin.

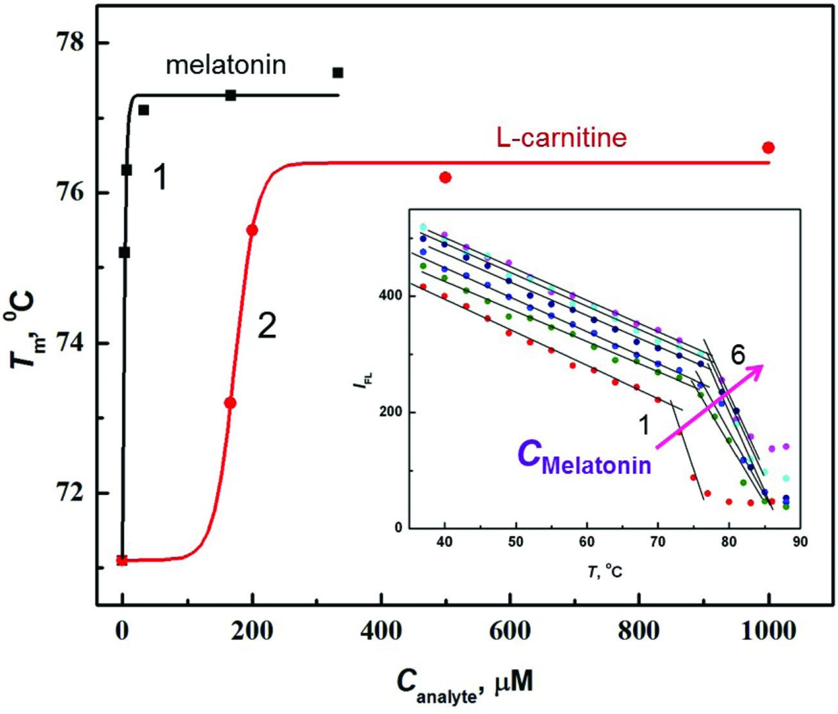


Figure 9. Dependence of T_{mo} vs. concentrations of antioxidants: (1) melatonin and (2) L-carnitine, INSET: Effect of melatonin on temperature dependence of DAPI-probe fluorescence recorded after interaction of ctDNA with paraquat and H_2O_2 in TRIS-HCl buffer (20 mM, pH = 7.5), C_{Mel} [µM]: (1) 0, (2) 3.3, (3) 6.67, (4) 33.3, (5) 166.7, (6) 333.3; C_{ctDNA} = 2.023 µM bp; C_{DAPI} = 166.7 nM; C_{PQ} = 100 µM, C_{H2O2} = 166.7 µM; ; t_{inc} = 1 h in 37 °C.

With increasing concentration of melatonin, C_{Mel}, a shift of T_{mo} towards $T_{mo,}$*, characteristic for an undamaged ctDNA, was observed. The value of the melting-onset temperature of ctDNA in the presence of L-carnitine antioxidant also shifted upwards from 71.1 °C to 76.6 °C. The relationship of melting-onset

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

temperature (T_m) vs. concentration of melatonin and L-carnitine was modeled using Boltzman function. It is seen that the addition of melatonin enhanced the protection of DNA against damage in the lower range of concentration than L-carnitine. 30 μM concentration of melatonin caused an increase of T_{mo} and prevented damage that would otherwise be induced by PQ. The protection by L-carnitine was also observed but at a higher concentration of 250 μM at which the value of T_{mo} measured was 76.6 °C. The results obtained above indicate that the addition of melatonin and L-carnitine has reduced and even prevented DNA damage by radicals.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

3.5. Molecular Dynamics Simulation of the Interaction of PQ with dsDNA

To evaluate the intercalation process of PQ into DNA duplex, molecular dynamics simulations and quantum mechanical calculation of electronic structure have been carried out. The interaction of herbicide with model double-stranded DNA consisting of 11 nucleic base pairs is presented in Figure 10.

The molecular dynamics of the intercalation of PQ was initiated by placing 3 PQ molecules between bases in the model of DNA molecule used for simulation. It is seen, that the interbase distance between bases and the DNA torsion dynamic change after interaction of DNA with PQ. This modeling provided clear evidence of conformational alterations in the DNA structure caused by the intercalation process. Clearly, paraquat causes unwinding of the DNA until the denaturation of double stranded helix to single stranded DNA occurs. These results of molecular dynamics simulation corroborate earlier conclusions of the substantially increased interbase distance and longitudinal duplex expansion due to the intercalation of PQ and are consistent with our results obtained from cyclic voltammetric and fluorimetric measurements. Similar data were obtained by our group after interactions of DNA with another herbicide, atrazine (Atz) (*4*). After longer time of DNA interaction with PQ, the DNA cleaving is observed.

In Figure 11, the results of quantum mechanical calculations for PQ, Mel and H_2O_2 are presented. We have found that melatonin forms hydrogen bonds with H_2O_2 but not with PQ. In absorbance measurements, we have not observed any strong interactions of Mel with PQ that would cause significant changes in the absorbance spectra obtained for experimental and theoretical data. It confirms that the prevention of DNA damage induced by PQ in the presence of H_2O_2 was caused by interactions of melatonin with hydrogen peroxide. We have found that melatonin forms with H_2O_2 strong hydrogen bonds and one melatonin molecule is able to bind directly up to 6 H_2O_2 molecules. Thus the experimental value of the critical H_2O_2/melatonin concentration ratio for the effective DNA-protection: $r_{exp} = 167/30 \approx 5.5$ is close to the number of H_2O_2 molecules directly bound to melatonin. Other H_2O_2 molecules can form a tight network of H-bonded molecules making melatonin a highly effective antioxidant that inactivates H_2O_2. The critical ratio of H_2O_2/melatonin is in perfect agreement with the results of quantum mechanical calculations ($r_{theor} = 6$).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

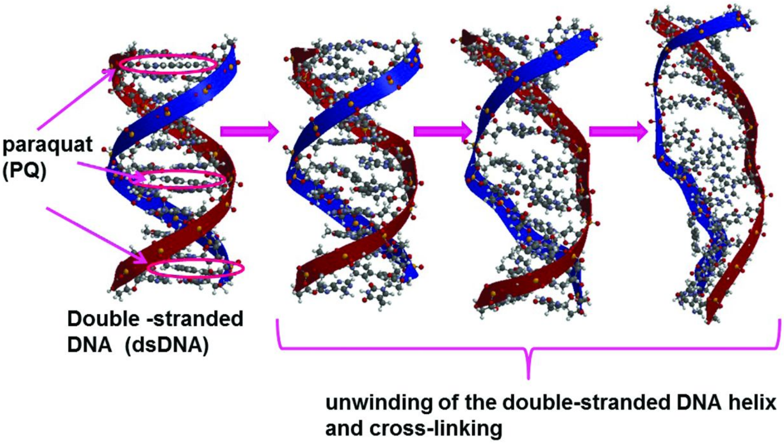


Figure 10. Molecular dynamics simulation of the interaction of paraquat (PQ) with double-stranded ctDNA (dsDNA).

3.6. Mechanism of PQ-Mediated ROS Generation in the Presence of H_1O_2

Yamamoto and Mohanan have reported (*21*) that the incubation of brain or lung tissue with PQ and ctDNA results in a PQ-mediated DNA damage. We have obtained similar results for a PQ-mediated ctDNA damage in the presence of H_2O_2, with DNA damage increasing with the PQ concentration (Figure 8). The difference between a biological system (*in vivo* and *in vitro*) and our model system is the presence of enzymes and redox-active biomolecules (like NADH, GSH, cysteine, redox proteins, etc.) that can reduce PQ to a radical form which then induces promptly the ROS formation and leads to significant DNA damage. Much less DNA damage is observed in model experiments where only PQ and DNA are present. Hence, usually, metal ions (like Fe(II), Cu(II)) are employed in a catalytic PQ-mediated ROS generation. In this work, we have utilized H_2O_2 instead.

Since PQ is introduced to the solution in the oxidized state (as a methyl viologen, MV^{2+}), it does not generate a $HO^{\bullet}$ radical in a direct reaction with H_2O_2. As shown below, the formation of a superoxide $O_2^{\bullet-}$ radical and the paraquat radical $MV^{\bullet+}$ in a reaction of paraquat with hydrogen peroxide is the critical step in the ROS generation. Once the paraquat radical $MV^{\bullet+}$ is formed, it can interact with H_2O_2 in a Fenton-like process to produce a $HO^{\bullet}$ radical:

$$MV^{2+} + H_2O_2 = MV^{\bullet+} + O_2^{\bullet-} + 2H^+$$

$$H_2O_2 + O_2 = 2O_2^{\bullet-} + 2H^+$$

$$MV^{2+} + O_2^{\bullet-} = MV^{\bullet+} + O_2$$

$$MV^{\bullet+} + H_2O_2 = MV^{2+} + HO^{\bullet} + OH^{-}$$

The formation of a hydroxyl radical $HO^{\bullet}$, the most powerful ROS, is the key element in the ROS generation. Once $HO^{\bullet}$ radicals have formed and reached DNA, they can quickly react with nitrogen bases and deoxyribose leaving oxidized bases and strand breaks (Dahm-Daphi et al. (*52*)).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

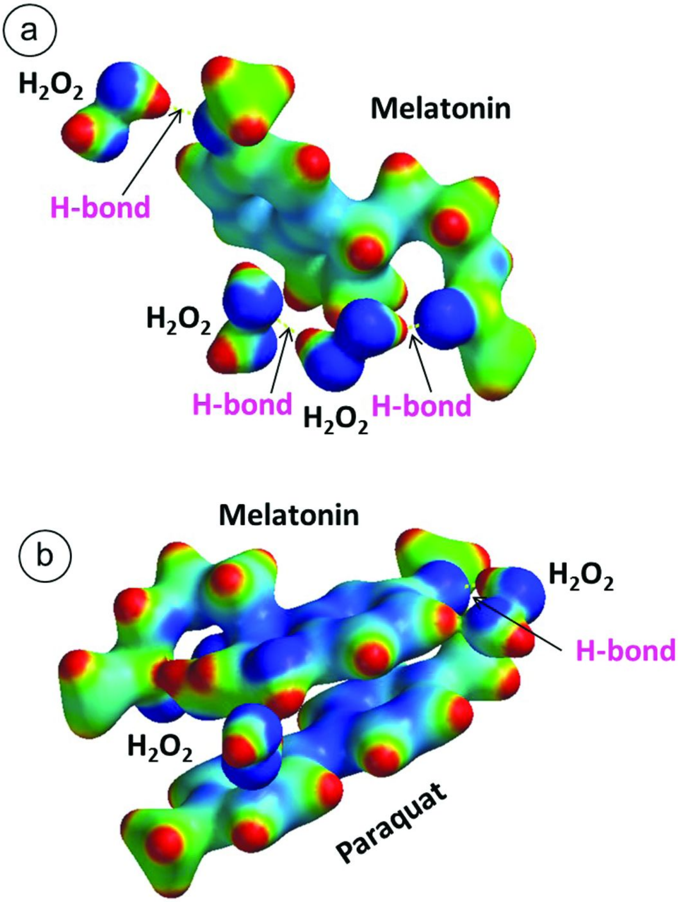


Figure 11. Electronic structures of: (a) melatonin + $3H_2O_2$ system and (b) melatonin + $2H_2O_2$ + paraquat system, calculated using quantum mechanical DFT method; electron density surfaces for $\rho = 0.08$, with electrostatic potential mapping (color coded, from negative potential - blue, to positive potential - red).

4. Conclusions

The DNA damage caused by paraquat in the presence of H_2O_2 has been investigated. It follows from our experiments and literature data that PQ alone can cause some DNA damage (*e.g.* Figures 7 and 8). Hydrogen peroxide is ubiquitous in biological systems and under physiological conditions, at low concentration, does not cause any considerable damage to DNA. However, in the presence of PQ, the H_2O_2 generates ROS and induces a significant DNA damage. This is clearly seen in Figure 7 where the DNA melting data are compared in the absence of PQ, in the presence of PQ alone, and in the presence of PQ + H_2O_2. The reaction mechanism involved in the ROS generation, including the formation of a highly reactive $HO^{\bullet}$ radical, has been elucidated. We have demonstrated using two methods, electrochemical biosensor method and fluorescence-probe DNA-melting method, that the DNA damage caused by paraquat in the presence of H_2O_2 is extensive but can be controlled with antioxidants melatonin and L-carnitine. These antioxidants are able to cross the brain barrier, so they can also function in the brain which is important for treatment of such diseases as the Down syndrome, autism, end-stage renal failure, and others where considerable oxidative stress is involved. We have found that the the DNA-protecting efficiency of melatonin is higher than that of L-carnitine. The critical concentrations of these antioxidants are equal to 30 μM and 250 μM, respectively, for a DNA-damaging medium consisting of a 100 μM PQ + 167 μM H_2O_2 used in this study. The mechanism of ROS generation by PQ is not completely understood. PQ is normally in its oxidized state, as a dication, and cannot directly interact with molecular oxygen. It requires a reducing species to form a $PQ^{\bullet+}$ cation radical which can trigger the ROS generating cascade. Apparently, H_2O_2 can reduce PQ^{2+} to $PQ^{\bullet+}$ and thus H_2O_2 is essential for the ROS production. Antioxidants that strongly interact with H_2O_2 can inhibit ROS generation by inactivating H_2O_2. We have found that melatonin forms with H_2O_2 strong hydrogen bonds and one melatonin molecule is able to bind directly up to 6 H_2O_2 molecules. Thus the experimental value of the critical H_2O_2/melatonin concentration ratio for the effective DNA-protection, $r_{\text{exp}} = 167/30 \approx 5.5$, is close to the number of H_2O_2 molecules directly bound to melatonin. Hence, the critical ratio of H_2O_2/melatonin is in perfect agreement with the results of quantum mechanical calculations ($r_{\text{theor}} = 6$). In comparison to melatonin, L-carnitine which is a smaller molecule can bind less H_2O_2 molecules (up to 4) but can inactivate only one of them. This makes melatonin a more effective preventor of PQ-mediated DNA damage in the presence of H_2O_2.

Acknowledgments

This work was partially supported by the U.S. DoD grant No. AS073218.

References

1. Oikawa, S.; Kawanishi, S. Distinct mechanisms of site-specifc DNA damage induced by endogenous reductants in the presence of iron(III) and copper(II). *Biochim. Biophys. Acta* **1998**, *1399*, 19–30.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

2. Battin, E. E.; Brumaghim, J. L. Metal specificity in DNA damage prevention by sulfur antioxidants. *J. Inorg. Biochem.* **2008**, *102*, 2036–2042.
3. Nowicka, A. M.; Kowalczyk, A.; Stojek, Z.; Hepel, M. Nanogravimetric and voltammetric DNA-hybridization biosensors for studies of DNA damage by common toxicants and pollutants. *Biophys. Chem.* **2010**, *146*, 42–53.
4. Hepel, M.; Stobiecka, M., *Interactions of Herbicide Atrazine with DNA*; Nova Science Publishers, Inc.: New York, 2010.
5. Melchiorri, D.; Ortiz, G. G.; Reiter, R. J.; Sewerynek, E.; Daniels, W. M. U.; Pablos, M. I.; Nistico, G. Melatonin reduces paraquat-induced genotoxicity in mice. *Toxicol. Lett.* **1998**, *95*, 103–108.
6. Hafez, A. M. Antigenotixic activity of melatonin and selenium against genetic damage induced by paraquat. *Aust. J. Basic Appl. Sci.* **2009**, *3*, 2130–2143.
7. Gulcin, I. Antioxidant and antiradical activities of L-carnitine. *Life Sci.* **2006**, *78*, 803–811.
8. Ribas, G. S.; Manfredini, V.; de Marco, M. G.; Vieira, R. B.; Wayhs, C. Y.; Vanzin, C. S.; Biancini, G. B.; Wajner, M.; Vargas, C. R. Prevention by L-carnitine of DNA damage induced by propionic and L-methylmalonic acids in human peripheral leukocytes in vitro. *Mutat. Res.* **2010**, *702*, 123–128.
9. Berni, A.; Meschini, R.; Filippi, S.; Palitti, F.; De Amicis, A.; Chessa, L. L-carnitine enhances resistance to oxidative stress by reducing DNA damage in *Ataxia telangiectasia* cells. *Mutat. Res.* **2008**, *650*, 165–174.
10. Santoro, A.; Lioi, M. B.; Monfregola, J.; Salzano, S.; Barbieri, R.; Ursini, M. V. L-carnitine protects mammalian cells from chromosome aberrations but not from inhibition of cell proliferation induced by hydrogen peroxide. *Mutat. Res.* **2005**, *587*, 16–25.
11. Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915–7922.
12. Haripriya, D.; Sangeetha, P.; Kanchana, A.; Balu, M.; Panneerselvam, C. Modulation of age-associated oxidative DNA damage in rat brain cerebral cortex, striatum and hippocampus by L-carnitine. *Exp. Gerontol.* **2005**, *40*, 129–135.
13. Melchiorii, D.; Reiter, R. J.; Sewerynek, E.; Hara, M.; Chen, L.; Nistico, G. Paraquat toxicity and oxidative damage reduction by melatonin. *Biochem. Pharm.* **1996**, *51*, 1095–1099.
14. Chen, Q.; Niu, Y.; Zhang, R.; Guo, H.; Gao, Y.; Li, Y.; Liu, R. The toxic influence of paraquat on hippocampus of mice: Involvement of oxidative stress. *NeuroToxicology* **2010**, *31*, 310–316.
15. Peng, J.; Stevenson, F. F.; Hsu, M.; Andersen, J. K. The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. *J. Biol. Chem.* **2004**, *279*, 32626–32632.
16. Shimizu, K.; Matsubara, K.; Ohtald, K.; Saito, O.; Shiono, H. Paraquate induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. *Brain Res.* **2003**, *976*, 243–52.
17. Ali, S.; Jain, S. K.; Abdulla, M.; Athar, M. Paraquat induced DNA damage by reactive oxygen species. *Biochem. Mol. Biol. Int.* **1996**, *67*, 63–67.

18. Dinis-Oliveira, R. J.; Remiao, F.; Carmo, H.; Duarte, J. A.; Sanchez Navarro, A.; Bastos, M. L. Paraquat exposure as an etiological factor of Parkinson's disease. *Neurotoxicology* **2006**, *27*, 1110–1122.
19. Di Monte, D. A.; Lavasani, M.; Manning-Bog, A. B. Environmental factors in Parkinson's disease. *Neurotoxicology* **2002**, *23*, 487–502.
20. Nagano, N.; Yagi, M.; Nishikori, K. Protective effects of antioxidants on paraquat-induced acute renal failure in mice. *Jpn. J. Pharmacol.* **1992**, *59*, 481–483.
21. Yamamoto, H.; Mohanan, P. V. Effects of melatonin on paraquat or ultraviolet light exposure-induced DNA damage. *J. Pineal Res.* **2001**, *31*, 308–313.
22. Caporossi, D.; Ciafre, S. A.; Pittaluga, M.; Savini, I.; Farace, M. G. Cellular responses to H_2O_2 and bleomycin-induced oxidative stress in L6C5 rat myoblasts. *Free Radical Biol. Med.* **2003**, *35*, 1355–1364.
23. Schmuck, G.; Rohrdanz, E.; Tran-Thi, Q. H.; Kahl, R.; Schluter, G. Oxidative stress in rat cortical neurons and astrocytes induced by paraquat in vitro. *Neurotoxic. Res.* **2002**, *4*, 1–13.
24. Yang, W.; Tiffany-Castiglioni, E. The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: Relevance to the dopaminergic pathogenesis. *J. Toxicol. Environ. Health, Part A* **2005**, *68*, 1939–1961.
25. Mangano, E.; Hayley, S. Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: Neuroimmune sensitization of neurodegeneration. *Neurobiol. Aging* **2008**, *30*, 1361–1378.
26. Ross, W. E.; Block, E. R.; Chang, R.-Y. Paraquat-induced DNA damage in mammalian cells. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 1302–1308.
27. Togunaga, I.; Kubo, S.-i.; Mikasa, H.; Suzuki, Y.; Morita, K. Determination of 8-hydroxy-deoxyguanosine formation in rat organs: assessment of paraquat-evoked oxidative DNA damage. *Biochem. Mol. Biol. Int.* **1997**, *43*, 73–77.
28. Wu, L. L.; Chiou, C. C.; Chang, P. Y.; Wu, J. T. Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetes. *Clin. Chim. Acta* **2004**, *339*, 1–9.
29. Stobiecka, M.; Hepel, M., Nitrotyrosine as a Biomarker of Diabetes. In *Advances in Medicine and Biology*; Berhardt, L. V., Ed. Nova Science Publishers, Inc.: New York, 2011; Vol. 13, pp 177−202.
30. Chagas, R. M.; Silveira, J. A. G.; Ribeiro, R. V.; Vitorello, V. A.; Carrer, H. Photochemical damage and comparative performance of superoxide dismutase and ascorbate peroxidase in sugarcane leaves exposed to paraquat-induced oxidative stress. *Pestic. Biochem. Physiol.* **2008**, *90*, 181–188.
31. Sliwinski, T.; Rozej, W.; Morawiec-Bajda, A.; Morawiec, Z.; Reiter, R.; Blasiak, J. Protective action of melatonin against oxidative DNA damage – Chemical inactivation versus base-excision repair. *Mutat. Res.* **2007**, *634*, 220–227.
32. Ross, J. H.; Krieger, R. I. Structure –activity correlations of amines inhibiting active uptake of paraquat (methyl viologen) into rat lung slices. *Toxicol. Appl. Pharmacol.* **1981**, *59*, 238–249.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

33. Muthuswamy, A. D.; Vedagiri, K.; Ganesan, M.; Chinnakannu, P. Oxidative stress-mediated macromolecular damage and dwindle in antioxidant status in aged rat brain regions: Role of L-carnitine and DL-α-lipoic acid. *Clin. Chim. Acta* **2006**, *368*, 84–92.
34. Alzahrani, H. A. S. Protective effect of L-carnitine against acrylamide-induced DNA damage in somatic and germ cells of mice. *Saudi J. Biol. Sci.* **2011**, *18*, 29–36.
35. Resnick, A. Z.; Kagan, V. E.; Ramsey, R.; Tsuchiya, M.; Khwaya, S.; Serbinova, E. A.; Packer, R. Antiradical effects in L-propionyl carnitine protection of the hearth agaisnt ischemia-reperfusion injury: The possible role iron chelation. *Arch. Biochem. Biophys.* **1992**, *296*, 394–401.
36. Vescovo, G.; Ravara, B.; Gobbo, V.; Sandri, M.; Angelini, A.; Della-Barbera, M.; Dona, M.; Peluso, G.; Calvani, M.; Mosconi, L.; Dalla-Libera, L. L-carnitine: A potential treatment for blocking apoptosis and preventing skeletal muscle mypathy in hearth failure. *Am. J. Physiol. Cell Physiol.* **2002**, *283*, 802–810.
37. Singh, R. B.; Niaz, M. A.; Agarwal, P.; Beegum, R.; Rastogi, S. S.; Sachan, D. S. A randomized, double-blind, placebo-controlled trial of L-carnitine in suspected acute myocardial infaction. *Post-Grad. Med. J.* **1996**, *72*, 45–50.
38. Binienda, Z.; Ali, S. F. Neuroprotective role of L-carnitine in the 3-nitropropionic acid induced neurotoxicity. *Toxicol. Lett.* **2001**, *125*, 67–73.
39. Vanella, A.; Russo, A.; Acquaviva, R.; Campisi, A.; Giacomo, G. D.; Sorrenti, V.; Barcellona, M. L. L-propionyl-carnitine as superoxide scavenger, antioxidant, and cleavage protector. *Cell Biol. Toxicol.* **2000**, *16*, 99–104.
40. Lorenti-Garcia, C.; Filippi, S.; Mosesso, P.; Calvani, M.; Nicolai, R.; Mosconi, L.; Palitti, F. The protective effect of L-carnitine in peripheral blood human lymphocytes exposed to oxidative agents. *Mutagenesis* **2006**, *21*, 21–27.
41. Boerrigter, M.; Franceschi, C.; Arigoni-Martelli, E.; Wei, J. Y.; Vijg, J. The effect of L-carnitine and acetyl-L-carnitine on the disappearance of DNA single-strand breaksin human peripheral blood. *Carcinogenesis* **1993**, *14*, 2133–2136.
42. Hepel, M.; Stobiecka, M. Novel DNA-Hybridization Biosensors for Studies of Atrazine Interactions with DNA. In *Advances in Environmental Research*; Daniels, J. A., Ed. Nova Science Publishers, Inc.: New York, 2010; Vol. 6.
43. Hepel, M.; Stobiecka, M. *Interactions of Herbicide Atrazine with DNA*; Nova Science Publishers, Inc.: New York, 2010.
44. Hepel, M.; Stobiecka, M. Detection of Oxidative Stress Biomarkers Using Functional Gold Nanoparticles. In *Fine Particles in Medicine and Pharmacy*; Matijevic, E., Ed.; Springer: New York, 2011.
45. Minchin, R. F. Evidence for the reversible binding of paraquat to deoxyribonucleic acid. *Chem.-Biol. Interact.* **1987**, *61*, 139–149.
46. Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Strekowski, L.; Boykin, D. W. Binding of 4′,6-diamidino-2-phenylindole (DAPI) to GC and mixed

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

sequences in DNA: Intercalation of a classical groove-binding molecule. *J. Am. Chem. Soc.* **1989**, *111*, 5008–5010.

47. Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Jones, R. L.; Fox, K.; Wydra, R. L.; Strekowski, L. DNA sequence dependent binding modes of 4′,6-diamidino-2-phenylindole (DAPI). *Biochemistry* **1990**, *29*, 8452−8461.
48. Härd, T.; Fan, P.; Kearns, D. R. A fluorescence study of the binding of Hoest 33258 and DAPI to halogenated DNAs. *Photochem. Photobiol.* **1990**, *51*, 77–86.
49. Kubota, Y.; Kubota, K.; Tani, S. DNA binding properties of DAPI (4′,6-diamino-2-phenylindole) analogs having an imidazole ring or a tetrahydropyrimidine ring: Groove-binding and intercalation. *Nucleic Acids Symp. Ser.* **2000**, *44* (1), 53–54.
50. Pineda De Castro, L. F.; Zacharias, M. DAPI binding to the DNA minor groove: A continuum solvent analysis. *J. Mol. Recognit.* **2002**, *15*, 209–220.
51. Banerjee, D.; Pal, S. K. Dynamics in the DNA recognition by DAPI: Exploration of the various binding modes. *J. Phys. Chem. B* **2008**, *112*, 1016–1021.
52. Dahm-Daphi, J.; Sass, C.; Alberti, W. Comparison of biological effects of DNA damage induced by ionizing radiation and hydrogen peroxide in CHO cells. *Int. J. Radiat. Biol.* **2000**, *76*, 67–75.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch007

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

Chapter 8

Artificial Nanoparticle Antioxidants

Erica Sharpe, Daniel Andreescu, and Silvana Andreescu*

**Department of Chemistry and Biomolecular Science,
Clarkson University, Potsdam, New York 13699-5810
*E-mail: eandrees@clarkson.edu**

Nanoparticle antioxidants constitute a new wave of antioxidant therapies for disease prevention and treatment in the field of oxidative stress. These 'artificial' antioxidants have demonstrated free radical scavenging activity, reducing the concentration of reactive oxygen and nitrogen species, thus acting as antioxidants by themselves. This review chapter discusses recent advances in the preparation, characterization and functionalization of these artificial nanoparticle antioxidants, the mechanism of their antioxidant activity and their use in the field of oxidative stress. These artificial antioxidants include inorganic nanoparticles possessing intrinsic antioxidant properties and nanoparticles functionalized with natural antioxidants or antioxidant enzymes to perform as an antioxidant delivery system. The antioxidant properties of these nanoparticles are discussed in relation to the structure, physicochemical properties and composition of these particles, parameters that are responsible for their redox activity and antioxidant action. Toxicity and potential applications of nanoparticles with antioxidant properties are also discussed. Antioxidant nanoparticles must be used with careful awareness of all their properties in order to achieve therapeutic results and balance antioxidant versus deleterious pro-oxidant effects.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

1. Introduction

Antioxidants have drawn much attention in recent years, due to their ability to fight oxidative stress, which has been linked to cancer, aging, and a wide variety of neurodegenerative diseases. Oxidative stress is created by highly reactive oxygen (ROS) and nitrogen (RNS) species, which are induced by environmental stress (e.g. ionizing radiation, redox and heavy metals, excess UV-radiation) or metabolism (e.g. secretion by macrophages, as well as by-product formation by the electron transport chain). ROS and RNS cause damage to lipids, cell membranes, proteins and DNA, leading to mutations, apoptosis, and failure within these systems. The main function of an antioxidant is to prevent and repair the effects of ROS and RNS, which are thought to be one of the causes of diseases such as atherosclerosis, Alzheimer's and Parkinson's, along with many cancers and other resulting effects of aging. Antioxidants function through two primary pathways: *prevention* of ROS/RNS formation, and *scavenging* or neutralization of ROS/RNS (*1*). Antioxidants like quercetin, and citric acid have been found to have significant reducing properties, preventing molecular mechanisms that are responsible for ROS formation (*2*, *3*). A wide variety of antioxidants have been seen to primarily function as scavengers of existing free radicals (*4*). Other antioxidants, primarily enzymatic compounds, are responsible for decomposition of reactive oxygen/nitrogen species into less harmful or neutral products (*4*).

An emerging area of research in the field of oxidative stress is the use of nanoparticles, possessing redox properties, which act as effective free radical scavengers. Several recent studies demonstrated that several types of nanoparticles have antioxidant properties (*5*, *6*). These 'artificial' antioxidants can be engineered at the nanoscale level (1-100 nm), have very high reactivity and can act as antioxidants by themselves. The antioxidant capacity depends on the nature, chemical composition, surface charge, surface to volume ratio and surface coating of the inorganic nanoparticles. A number of studies indicated that several redox active nanoparticles such as ceria and yttria act by mimicking the activity of oxidative enzymes, superoxide dismutase (SOD) and catalase (CAT) (*7*, *8*). Available literature data show that many of these nanoparticulate antioxidant systems protect cells against oxidative stress and are relatively nontoxic to cultured cells. On the other hand, inert nanoparticles such as gold can be used as carriers for antioxidant molecules. In this case, natural antioxidants can be attached onto the nanoparticle surface, providing a new class of supported antioxidants with increased antioxidant capacity. Using this strategy, it is possible to enhance efficiency and provide targeted delivery of certain natural antioxidants that are difficult to cross cell membranes and be internalized into cells. Such small particles of several nanometers are able to enter cells and cross cell membrane through pinocytosis. Supported antioxidants can be delivered into the cytoplasm and mitochondria where they can neutralize ROS and RNS. As an example, nanoparticles loaded with glutathione (GSH) have been shown to penetrate cell membranes and to successfully deliver and release drug molecules in living cells (*9*). Recent developments in this field demonstrate the potential of this technology for designing new powerful antioxidants based on inorganic nanoparticles for disease prevention and therapy.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

This review chapter provides an overview of the different classes of antioxidant nanoparticles derived from inorganic materials including those with intrinsic antioxidant capacity and those that can be functionalized to become potent antioxidants. The chapter discusses recent advances in the preparation, characterization and functionalization of these particles, the mechanism of their antioxidant activity and their use in the field of oxidative stress. The antioxidant properties of these nanoparticles are discussed in relation to their structure, physicochemical properties and composition; parameters that are responsible for their redox activity and antioxidant action. Toxicity and physiological effects of nanoparticles with antioxidant properties are also discussed.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

2. Classes of Nanoparticle Antioxidants

2.1. Inorganic Nanoparticles with Intrinsic Antioxidant Properties as Free Radical Scavengers

Several types of inorganic nanoparticles have demonstrated intrinsic free radical scavenging activity, decreasing concentrations of ROS and RNS, and thus acting as antioxidants (*10*). Their effectiveness in mitigating oxidative stress has been demonstrated in complex biological systems including cell cultures and in *in vivo* conditions. This interesting characteristic has been related to their redox and catalytic properties, electronic configuration, high surface-to-volume ratio and biocompatibility. Therefore, potential use of these nanoparticles to reduce oxidative stress in biological systems and treat oxidative stress related diseases is increasingly being explored. Classes of inorganic nanoparticles possessing intrinsic antioxidant activity range from metal (gold and platinum) to several types of metal oxides (e.g. ceria, yttria, and iron oxide).

Gold, platinum and nickel oxide (*11*, *12*) have been found to have ROS neutralizing abilities. Platinum has been found to be particularly effective in reducing smoke-induced respiratory inflammation in the respiratory system, which has been associated with high levels of ROS and depletion of the normal antioxidant function (*13*, *14*). Experimental results showed that mice, which received platinum nanoparticles stabilized with poly(acrylate) prior to exposure to smoke, experienced less inflammation and decreased cell death than those in a control group which received a saline solution. Platinum nanoparticles (2.4 nm) have also been shown to scavenge superoxide and hydrogen peroxide in a dose-dependent manner, and to prolong the lifespan of *Caenorhabditis elegans* (*12*). In another study, 50 nm gold nanoparticles were found to inhibit oxidative stress mediated diabetic progression during hyperglycemia in mice, demonstrating therapeutic potential in diabetic treatment (*15*). No toxic effects were observed when the mice were injected with the particles at a dosage of 2.5 mg/kg.b.wt/day for 15 days. The mechanism of action has been attributed to either the inhibition of the stress signaling pathways or to the interaction of gold with the cysteine residues of a thioreductase, thioredoxin, which is involved in the antioxidative mechanism in hyperglycemic conditions. Chitosan-stabilized gold nanoparticles with diameters ranging between 6 and 16 nm have shown antioxidant action against hydroxyl radicals formed in a hydrogen peroxide/$FeSO_4$ system (*16*).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

In addition to gold and platinum, nickel oxide nanoparticles have been shown to have radical scavenging properties through in vitro testing using the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity assay. However, at high doses, nickel is toxic and can have adverse cytotoxic effects when used in a biological environment. In other studies, iron oxide nanoparticles have been shown to possess antioxidant properties (*17*). Diamond nanoparticles (4 nm diameter) decorated with gold and platinum nanoparticles (<2 nm diameter) exhibited high antioxidant activity against ROS in a hepatoma cell line (*18*). Figure 1A shows the decomposition profile of hydrogen peroxide by the gold and platinum decorated diamond. The gold decorated diamond was two-fold more active against induced oxidative stress in cells than GSH, a reference antioxidant (Figure 1 B).

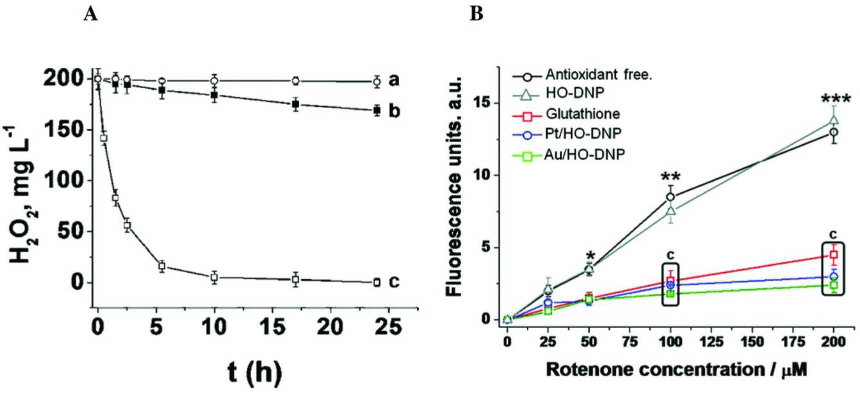


*Figure 1. **A**: Decomposition of 5.88 mM H_2O_2 by diamond nanoparticles (a) and by gold (b) and platinum (c) decorated diamond nanoparticles. **B**: Antioxidant effect of diamond nanoparticles (HO-DNP), glutathione and gold (Au/HO-DNP), (b) and platinum (Pt/HO-DNP), (c) decorated diamond nanoparticles on rotenone-induced ROS production at different concentrations showing fluorescence decrease in Hep3B cells. From reference (18).*

Several other inorganic materials including cerium oxide (ceria or CeO_2) (*7*, *19*) , yttrium oxide (or yttria) (*10*), iron oxide (*20*) and manganous phosphate (*21*, *22*) have been shown to have the ability to mimic SOD and CAT, two essential antioxidant enzymes that are protecting cells against ROS. SOD and CAT function by catalyzing disproportionation reactions of their substrates, the superoxide radical and hydrogen peroxide. However, the mechanism of the enzyme-like activity of these particles is currently poorly understood. These properties have been used in the treatment of several oxidative diseases. For example, ceria and yttria nanoparticles have neuroprotective effects and demonstrated therapeutic efficacy in the treatment of neurodegenerative diseases. Both types of nanoparticles have been found to protect nerve cells from oxidative stress irrespective of particle size (*23*). We found evidence that the direct or indirect scavenging of peroxynitrite by ceria nanoaprticles contributes to its neuroprotective effects in an *in vitro* model of ischemia in organotypic brain

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

slices prepared from CD1 mice (*24*). In other studies, ceria protected cells which were exposed to H_2O_2, as a source of reactive oxygen molecules which would induce oxidative damage (*25*, *26*) . Similar effects were reported with yttria (*10*). As a result, ceria nanoparticles can be used as therapeutic agents in the treatment of medical diseases related to ROS. These include: (i) spinal cord repair as neuroprotective agents (*25*, *26*), (ii) modulation of oxidative stress in biological systems and promotion of cell survival in oxidative conditions (*10*, *27*), (iii) scavenging of reactive molecules in the eye, thus preventing degenerative retinal disorders (*27*), (iv) protection of gastrointestinal cells from radiation induced damage (*28*) and ischemia (*24*). In a recent study, the antioxidant effect of ceria nanoparticles (5-16 nm diameter) were studied on U937 and Jurkat cells (*29*). It was found that ceria decreased free radical formation as well as damage induced by apoptosis (Figure 2); the antioxidant effects correlated with the amount of Ce^{3+} but not with oxygen vacancies.

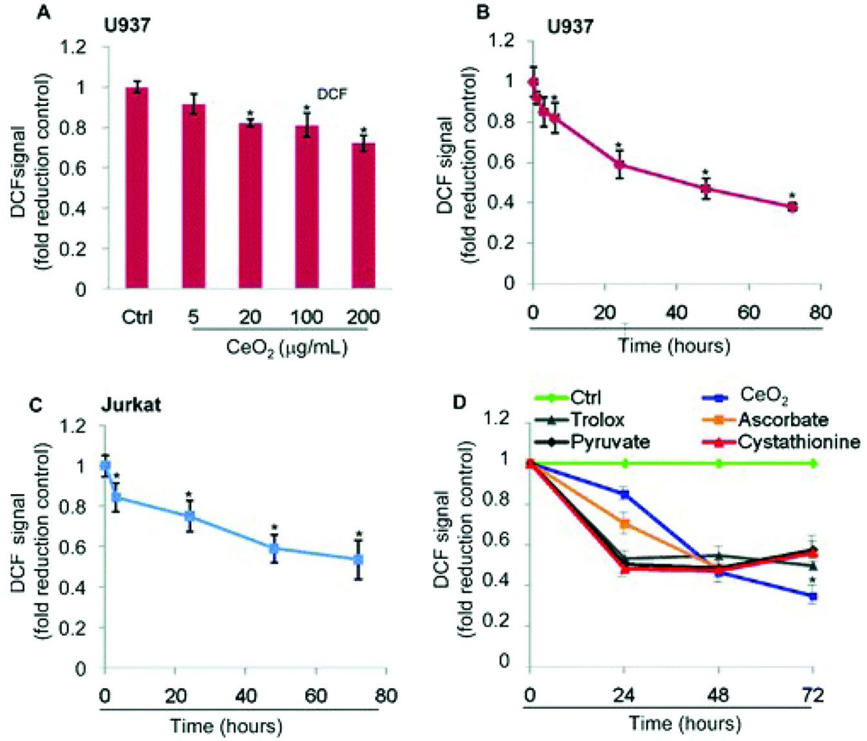


Figure 2. Ceria nanoparticles reduce intracellular ROS in U937 and Jurkat cells. ***A****: concentration dependency in U937.* ***B*** *and* ***C****: time dependency in U937 and Jurkat cells.* ***D****: Comparison of the radical scavenging activity of ceria (expressed as decrease of the fluorescence signal of dichlorofluorescein (DCF)) versus trolox, ascorbate, cystathione and pyruvate, showing progressive decrease of ROS levels in the presence of ceria, a time dependent manner, similar to that observed with the other antioxidants tested. From reference (29).*

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

Ceria is a material that is intensively used in heterogeneous catalysis, solid oxide fuel cells and in gas sensing. In biology and medicine it has received significant attention due to its catalytic and free radical scavenging properties, deactivating reactive oxygen species and making them non-reactive (*28*, *30*, *31*). The many useful catalytic properties of ceria have been attributed to the presence of highly mobile lattice oxygen present at its surface and a large diffusion coefficient of the oxygen vacancy which facilitates the conversion $Ce^{4+} \leftrightarrow Ce^{3+}$ and thus, allows oxygen to be released or stored in its cubic structure (*25*, *32*, *33*). An interesting observation is the regenerative behavior of these particles involving a reversible oxidation /reduction cycle (*6*). Changes in the oxidation state can occur as a result of various parameters such as presence of reactive species, temperature and pH (*7*, *34*). Such processes are determined by the reversible adsorption-desorption of chemisorbed oxygen, and reduction-oxidation of surface metal oxides. Their performance is determined by the composition of the oxide layers, the size, available surface area and the ratio Ce^{3+}/Ce^{4+} (*25*). Typically, nanocrystalline oxides present higher vacancy mobility compared to bulk oxides (*35*, *36*). Most studies to date have almost exclusively utilized simple ceria. Oxygen vacancies and surface processes can be modified if ceria is in contact with noble metals which can facilitate the transfer of oxygen from the bulk material to the surface and vice-versa (*36*, *37*). The local oxygen environment can also be modified by mixing or doping ceria with other rare-earth or transition metal oxides (*36*, *38*). Zirconia (ZrO_2), yttria (Y_2O_3) and titania (TiO_2) are such examples. One study reported that mixed CeO_2-ZrO_2 nanoparticles exhibit a greater redox and catalytic activity (*38*). These more complex oxides including binary and tertiary systems have been used almost exclusively for fuel cell applications, but such systems can prove to be effective antioxidants. These studies open up attractive opportunities for designing a new generation of antioxidants with enhanced efficacy.

2.2. Effect of Surface Chemistry and Physicochemical Characteristics: Surface Charge, Surface Coating, Particle Size, and Redox State

Surface chemistry and physicochemical properties including surface charge, surface coatings (e.g. presence of stabilizers and functional groups) and particle size determine cellular uptake, bioavailability and distribution, influencing the effectiveness of these nanoparticles in the body (*39*, *40*). For example, cerium oxide has been found to be effective over a wide range of sizes (*41*); however, particles with various zeta potentials have different cellular uptake, as well as protein adsorption properties. Patil et al. (*39*) studied the effect of electrostatic interactions and found that ceria particles with positive zeta potentials are more conducive to BSA adsorption, while negatively charged particles showed little or no adsorption and were more easily taken into lung cells. The antioxidant properties of iron oxide, assessed using the DPPH assay were found to increase with decreasing particles size (*20*). Nanoparticle agglomeration is another important parameter, especially for in vivo applications as this can greatly affect cellular uptake and mobility of the nanoparticles into the intracellular space and tissues. Ultrasonication of the nanoparticles before use enhanced the antioxidant activity by 18.96% (assessed using the DPPH scavenging assay) (*42*).

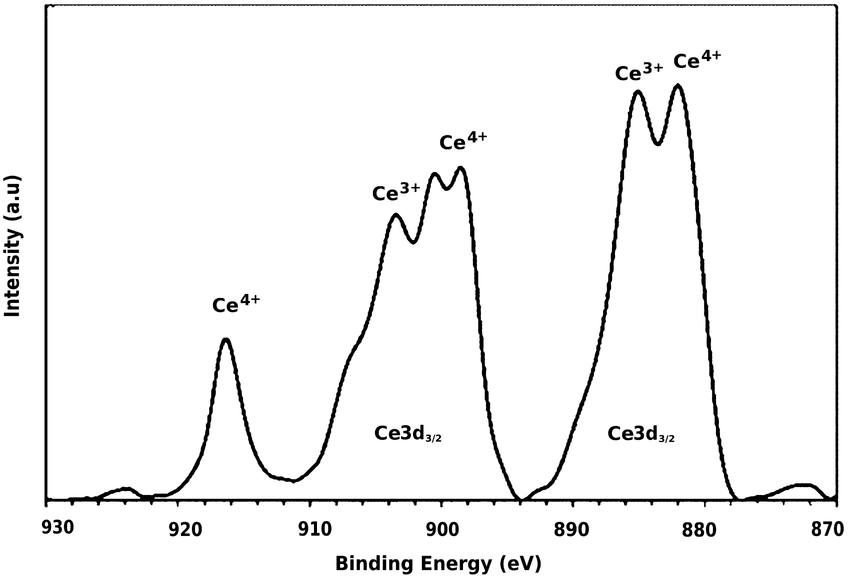


Figure 3. XPS (X-ray photoelectron spectroscopy) spectrum showing the presence of mixed Ce^{3+} and Ce^{4+} valence states on ceria nanoparticles of a diameter of 3-5 nm. From reference (25).

The redox state of the metal on the nanoparticle surface is another critical parameter that significantly impacts the ability of inorganic nanoparticles to react with ROS and RNS and act as antioxidants. This is because the redox processes take place at the surface of the nanoparticle (*7*). For example, the mechanism of the antioxidant activity of ceria is a consequence of the dual Ce^{3+}/Ce^{4+} oxidation state of cerium ions onto the nanoparticle surface (Figure 3) (*25*) and is directly related to Ce^{3+} concentrations at the nanoparticle surface (*25*, *29*). In dry colloidal state, at room temperature, cerium oxide is present in its more stable state, as CeO_2, in which ceria Ce^{4+} is the predominant species on the surface. Changes in the oxidation state, and subsequent changes in particle reactivity, can be made by various surface treatments. Thus the reactivity of the particles can be tuned by performing surface chemical reactions. In a study by Heckert et al. (*8*) X-ray photoelectron spectroscopy (XPS) and UV-Vis spectroscopy were used to show that when nanoceria was treated with hydrogen peroxide, a decreased ratio of reduced to oxidized cerium (Ce^{3+}/Ce^{4+}) was associated with loss of SOD mimetic activity and inactivation of superoxide radicals. Therefore, the surface oxidation state of ceria nanoparticles is not only responsible for its antioxidant activity, but is a very important parameter in defining other aspects of cerium oxide's activity (*29*). The coexistence of the Ce^{3+}/Ce^{4+} enable ceria to react catalytically with both superoxide and hydrogen peroxide (oxidation of Ce^{3+} and reduction of Ce^{4+})

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

and act as SOD and CAT mimetics (*7*, *43*). Thus, the ability of ceria to scavenge superoxide radicals depends on the existence of high concentrations of Ce^{3+} at the nanoparticle surface. In another study, ceria nanoparticles functionalized with polyethylene glycol (PEG) demonstrated efficient radical scavenging properties against hydrogen peroxide and superoxide radicals (*31*). Surface functionalization with PEG did not change the antioxidant ability as compared with bare ceria. The antioxidant activity and stability was related to the formation of a charge transfer complex with hydrogen peroxide on the nanoparticle surface.

2.3. Functionalized and Composite Inorganic Nanoparticle Antioxidants

2.3.1. Antioxidant Functionalized Nanoparticles: Attachment of Antioxidants to Nanoparticles for Enhanced Antioxidant Activity and Targeted Delivery

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

Another class of ‘artificial’ antioxidants is that of inorganic nanoparticles that are surface functionalized with antioxidants. Several studies have demonstrated that attachment of naturally occurring or synthetic antioxidants onto the surface of inert inorganic nanoparticle such as gold results in an enhanced radical-scavenging activity and bioavailability (*44*, *45*). Certain antioxidants, like Vitamin E (alpha-tocopherol), though effective in disease treatment and prevention, have low solubility and poor bioavailability. Attachment of such antioxidants to inorganic nanoparticles can improve solubility and allow targeted delivery, would lead to more optimal usage. Attachment of Trolox (a water soluble form of Vitamin E) to gold nanoparticles through self-assembly of thiol ligands onto gold (*45*) was shown to provide enhanced antioxidant activity of the antioxidant nanoparticle formulation; that was eight times higher than that of Trolox alone (the scavenging rate was assessed using the DPPH assay). Work is currently in progress to create GSH functionalized gold nanoparticles, with the effort to make this antioxidant more bioavailable and permeable through cell membranes (*9*). This direction of work is particularly relevant because it is highly difficult to uptake GSH through dietary sources. It is hypothesized that cells would be able to more easily uptake GSH-functionalized particles through pinocytosis. After cellular uptake, the particles can act as radical scavengers in the cytoplasm or travel into the mitochondria to inactivate ROS. Further developments of this promising strategy might lead to novel powerful antioxidant formulations. However, the bioavailability, metabolism and cytotoxicity of these new antioxidants functionalized nanoparticles need to be further studied (*45*).

2.3.2. Nanoencapsulated Antioxidants

In addition to the use of inorganic nanoparticles, antioxidants can be nanoencapsulated in polymeric materials for achieving enhanced delivery and bioavailability. For example, a poorly soluble and unstable natural antioxidant with known iron-binding properties, quercetin (*46*) was successfully encapsulated in a polylactide (PLA) polymeric network, for controlled delivery and release (*47*). This method was 97% efficient in encapsulating free quercetin. The capsules

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

gradually released quercetin in a controlled fashion, over 72 hours. In another study, quercetin-loaded-nanoparticles with a particle size of less than 85 nm were created by a nanoprecipitation technique using poly(vinyl alcohol) and Eudragit. These 'nanocarrier' antioxidants demonstrated increased superoxide radical scavenging activity and higher efficiency in preventing superoxide formation through lipid peroxidation than the free quercetin (*48*). Ascorbic acid derived antioxidant particles of 170 nm diameter were produced from ascorbic acid and chitosan by ionic gelation of chitosan with tripolyphosphate (TPP) anions (*49*). The particles were used for fast and sustained release of ascorbic acid. Other antioxidants have been converted and can be used in nanoparticulate form. A nanoparticle system of curcumin, an antioxidant with low bioavailability, showed improved cellular uptake and enhanced antioxidant properties as compared to free curcumin (*50*). Using similar procedures, antioxidant enzymes such as CAT and SOD could be encapsulated for controlled delivery.

2.3.3. Magnetically Responsive Antioxidant Nanocarriers

Antioxidant enzymes have been loaded onto magnetic nanoparticles for controlled delivery through magnetic guiding (*51*). Magnetic nanoparticles that have attracted attention for use in biomedical applications are generally iron oxides (Fe_2O_3 and Fe_3O_4). Various forms and sizes of magnetic nanoparticles are available and can be purchased from several companies, or they can be prepared using established synthetic procedures (*52–54*). Magnetically responsive antioxidant nanocarriers can provide therapeutic guiding of high concentrations of antioxidants to specific locations with elevated levels of ROS. Using magnetic field guidance and enzyme-loaded magnetic nanoparticles, antioxidant enzymes (such as SOD or CAT) can be guided to the suitable spot, while being protected, by the nanoparticle carrier, from proteolytic inactivation. Catalase-loaded magnetic nanoparticles showed rapid cellular uptake and provided increased resistance to oxidative stress damage, induced by hydrogen peroxide (*51*). The results of this study indicate that magnetic nanoparticles are reliable carriers for targeted antioxidant enzyme therapy. One suggested application was for the treatment of cardiovascular diseases (*51*), which is correlated with oxidative damage.

3. Evaluation of Antioxidant Capacity in Relation to Physicochemical Characteristics

The existing antioxidant activity assays can be used to assess the antioxidant capacity of 'artificial' antioxidant nanoparticles. However, because there is no universal validated method that can reliably measure free radicals, limitations of existing methods need to be considered. Assays for measuring antioxidant capacity, the chemistry behind these assays and their advantages and limitations have been reviewed (*55*). The most widely used are the ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay, the Trolox equivalent antioxidant activity (TEAC), the DPPH radical scavenging assay and the oxygen radical absorbance capacity (ORAC) assay, among others (*55*). Most available assays only assess the ability of an antioxidant to scavenge a limited number of radicals (e.g. ABTS, peroxyl, superoxide). Quantifying the antioxidant activity of some radicals such as the hydroxyl radical is difficult due to their extremely short lifetime. It is also important to consider that antioxidants, including the newly designed class of redox active inorganic nanoparticles, react differently with each radical. Depending on the oxidation state and surface properties, nanoparticles can have different specificities to scavenging of certain radicals over others. Therefore, assessing antioxidant capacity using multiple, complementary assays is needed as each method is based on a different methodology, uses different substrates and reaction conditions and has its own specificity. It is expected that results from multiple assays might show disagreement with respect to the capacity of these particles to scavenge antioxidants. Inorganic nanoparticles having different sizes and surface coatings can show high variability between methods due to the large number of variables, some originating from the synthesis and stabilization process of these particles, others from nanoparticle agglomeration and surface coating.

Few studies, which assess the antioxidant capacity of nanoparticle systems, in relation to their physicochemical properties, have been published. In one such study, the ‘oxidase-like activity’ of ceria coated with a polymeric layer was found to be dependent on the pH, the size of the nanoparticles and the thickness of the polymeric coating (*10*, *34*, *38*). The radical scavenging capacity of several oxide nanoparticles (Fe_3O_4 and $CoFe_2O_4$ and MnO_2) stabilized by a biocompatible polymer, alginate, has been determined using the TEAC assay and an amperometric method. With the exception of MnO_2, all other oxide-alginate composites have efficiently scavenged hydrogen peroxide (*56*).

Systematic studies are needed to identify parameters that affect the reactivity and specificity of these particles against different radicals, and also to establish experimental variables and physicochemical properties that determine their antioxidant capacity. In addition, more fundamental studies to elucidate the mechanism of their antioxidant action and the efficiency of these particles in biologically relevant conditions, their interaction with proteins, cells and tissues are also needed in order to fully explore their potential in the biomedical field and to reasonably assess possibility for specific uses as therapeutic agents.

4. Toxicity and Biodistribution

The toxicity and biodistribution of antioxidant nanoparticle systems are strongly related with the nature of these particles, the purity, crystallinity, polydispersity, surface coating, oxidation state at the surface and their physicochemical properties. The surface chemistry and particle size determines antioxidant capacity but also the toxicity, fate and transport of these particles, in biological systems. While many metal and metal oxides have been reported to possess antioxidant properties at certain concentration levels, several other studies have shown that these particles can induce ROS production (*57*) and have

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

adverse cytotoxic effects in vitro and in vivo. Thus some particles such as ceria are reported to both scavenge (*5*, *7*) and generate ROS (*57*).

The interactions of these particles with proteins, cells and tissues, their cellular uptake, toxicity and distribution across tissues (*58–64*) and ultimately their bioavailability are also dependent on the physicochemical parameters and surface coating. It is also important to consider that the cytotoxic response (and also the antioxidant properties) of metal and metal oxide nanoparticles are dictated and can be controlled by changing the surface chemistry. Modification of the nanoparticle surface provides advantages including: addition of chemically useful functionalities, prevention of nanoparticle agglomeration, minimization of toxic effects in physiological conditions and enhanced bioaccumulation. The use of these particles in biomedicine as therapeutic agents in the prevention and treatment of oxidative stress diseases require production of biocompatible nanoparticles and nanoparticles with biocompatible coatings to enhance biodistribution and reduce toxicity across cells and tissues. Procedures for “green” synthesis of nanoparticles have been developed and these can be applied to the preparation of antioxidant nanoparticles for biological and biomedical applications. Green synthesis and modification of nanoparticles with biocompatible layers such as polymeric hydrogels (*65*) can improve biocompatibility and accessibility to target cells and tissue locations while conserving their biologically useful properties.

Toxicological studies are important to assess health risks and deleterious side effects and identify the therapeutic usefully window of these particles. Ceria nanoparticles do not appear particularly toxic. For example concentrations of ceria between 5 and 10 µg/ml did not cause DNA damage to human epithelial cells (*66*). However, the complex biomolecular processes taking place at the interface between the inorganic material/coating and the surrounding biological environment (*67*, *68*) should be carefully studied before clinical applications are envisioned.

While many metal and metal oxides have been shown to display antioxidant properties, several reports have also indicated the possibility that these particles can induce formation of free radicals and thus amplify oxidative stress damage (*41*, *42*). Most of these processes are taking place through Fenton chemistry in reaction with H_2O_2. Induction of ROS production is associated with the reactivity of active species at the nanoparticle surface and it is strongly dependent on the redox state of the metal ions as well as the concentration of these particles. It was found that while low doses of antioxidant nanoparticles can scavenge free radicals, when they are used in high amounts they can also produce ROS. Both hydroxyl and superoxide radicals were produced in the presence of hydrogen peroxide by Ce^{3+} ions (cerium chloride) by a series of Fenton-like reactions, as demonstrated by electron paramagnetic resonance (EPR) and the ABTS assay (*69*). As opposed to free cerium ions, colloidal ceria nanoparticles can form surface complexes in reaction with hydrogen peroxide. XPS and FT-IR images have shown the presence of peroxyl groups (-OOH) on the ceria nanoparticle surface following interaction with hydrogen peroxide (*70*). In other studies, high-dose titania nanoparticles delivered to the abdominal cavity caused oxidative stress and injury in the brains of mice, a process associated with lipid peroxidation, decrease of the anti-oxidation capacity, release of nitric oxide and reduction of glutamic acid. (*71*). Other studies

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

suggest that silver nanoparticles may induce oxidative stress in the brain of a rat, causing impairment of memory function (*72*).

While several studies have found that gold nanoparticles have the ability to inactivate ROS (*15*, *16*), in other studies, gold nanoparticles (1.2 nm diameter) have been shown to trigger necrosis by oxidative stress and mitochondrial damage (*73*). Modification of the nanoparticles with antioxidants such as GSH and acetylcysteine reduced toxicity. In the same study, GSH capped nanoparticles of similar sizes did not induce oxidative stress. This demonstrates the importance of surface coatings, in addition to size dependency, in determining the toxicity of these nanoparticles. Li et al have studied the effect of Au nanoparticles on MRC-5 lung fibroblasts and found increased levels of hydroperoxide radicals which could react with proteins and induce DNA damage (*74*). In another study, AuNP with small diameters (~5 nm) caused lipid peroxidation. Oxidative stress and cytotoxicity induced by small Au nanoparticles were higher than those induced by particles with larger diameters (*75*). The discrepancy between results reported in the literature with different types of particles (e.g. gold, ceria), showing both antioxidant and pro-oxidant capacity is due to the use of different types of nanoparticles, biological models, experimental conditions and antioxidant assays. More systematic studies are needed to identify and predict what types of particles, physicochemical characteristics and experimental conditions in which these particles behave as potent antioxidants.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

5. Applications of Antioxidant Nanoparticles for Therapy and Disease Prevention

The role of antioxidants in prevention and treatment of a variety of diseases including cancer, heart diseases, diabetes, neurodegenerative diseases, immune deficiency, and aging in general has been demonstrated in multiple cases (*76*). Conventional therapies that have been explored include the use of naturally occurring antioxidants such as epigallocatechingallate, alpha-tocopherol (Vitamin E), ascorbic acid, (Vitamin C), lycopene, ellagic acid, genestein, quercetin and antioxidant enzymes like SOD, CAT and glutathione peroxidase. Most of these compounds are extracted from natural products, however, many are poorly bioavailable and have low efficiency due to instability, inefficient permeability and poor solubility. Inorganic antioxidant nanoparticles either functionalized to perform as an antioxidant delivery system, or possessing intrinsic antioxidant properties, constitute a new wave of antioxidant therapies for disease prevention and treatment. In either case, the need for carefully chosen and optimized delivery techniques for maximum efficiency is needed. Moreover, antioxidant nanoparticles must be used with careful awareness of all their properties in order to achieve therapeutic results and balance antioxidant versus deleterious pro-oxidant effects. This section provides selected examples of applications of antioxidant nanoparticles in biomedicine.

The most widely studied antioxidant nanoparticles are ceria, or nanoceria. Numerous studies have demonstrated the antioxidant properties of ceria and their potential applications for the treatment of a variety of diseases, e.g. neurological

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

disorders, aging, ischemia, spinal cord repair (*25*, *26*) degenerative retinal disorders (*27*), cardiac stress, and inflammation (*30*, *77*, *78*). The mechanism of the antioxidant function of ceria is attributed to the redox activity of the nanoparticle surface, which is governed by mixed valence states of Ce^{3+} and Ce^{4+}. The autocatalytic reversible oxidation-reduction ability indicates that ceria can neutralize a wide range of reactive oxygen and nitrogen species and has potential as a therapeutic antioxidant (*6*). Nanoceria has been synthesized in biocompatible media, which increase biocompatibility and its ability to work well *in vivo*. Most studies, which evaluate the antioxidant activity of ceria, have been performed *in vitro*, mostly on cell cultures. Cells exposed to ceria nanoparticles maintained viability and promote cell survival in oxidative conditions (*10*, *27*). In another study ceria protected gastrointestinal epithelium cells from radiation induced damage (*28*) and against progression of cardiac dysfunction by attenuating myocardial oxidative stress and inflammatory processes in transgenic mice (*79*). It was found that nanomolar concentrations of ceria nanoparticles act as antioxidants, are biocompatible, regenerative, and provide neuroprotection to adult rat spinal cord neurons (*18*). We found that treatment with ceria nanoparticles ameliorates ischemic injury in the hippocampus, a region of the brain particularly vulnerable to the effects of oxygen and glucose deprivation associated with stroke (*24*). The use of ceria nanoparticles in biomedicine, the antioxidant function in biological systems and biological mechanisms are discussed in detail in another chapter of this book. Applications of antioxidant nanoparticles for the treatment of diabetes have also been explained. In a recent study, gold nanoparticles (50 nm diameter) have inhibited ROS formation in hyperglycemic conditions. ICP-MS (inductively coupled plasma mass spectroscopy) analysis showed that the particles bioaccumulate in the vital organs with the greatest accumulation in the spleen (*15*).

6. Conclusions and Future Trends

Though the field of nanoparticle antioxidants is still in its infancy, future research could lead to a new wave of powerful antioxidants for disease prevention and therapy. The potential impact of these materials in the field of oxidative stress is large. However, the mechanism that determines these unique properties is poorly understood. In order to take advantage of the unique capabilities of these nanomaterials as antioxidants for a variety of biological and biomedical applications, a fundamental understanding of the nature and mechanism that drives their catalytic properties and biological activity in relation to the physicochemical parameters is needed. Systematic studies are necessary to elucidate the mechanism of the antioxidant activity of inorganic nanoparticles. A study of the surface chemistry and redox processes at the nanoparticle surface is critical in order to rationally design nanoparticle antioxidants as therapeutic agents for disease prevention and therapy. In addition, the toxicity of the nanoparticles needs to be studied in greater detail before future biomedical applications with these particles can be pursued.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

Future advances are expected through the integration of the therapeutic properties of antioxidants and the technically and physically appealing features of nanoparticle engineering in order to increase bioavailability, solubility and achieve positive effects on the human organism. Development of novel drug delivery systems for more efficient delivery of therapeutic antioxidants in nanoparticulate form, as well as design of novel antioxidant activity assays which can more accurately and reliably determine their antioxidant activity are needed. As more knowledge of structure-activity relationships is gained, rational engineering of more powerful antioxidant nanoparticles might be possible.

Acknowledgments

This material is based upon work supported by the National Science Foundation under Grant No. 0954919. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

References

1. Koltover, V. K. Antioxidant biomedicine: From free radical chemistry to systems biology mechanisms. *Russ. Chem. Bull., Int. Ed.* **2010**, *59* (1), 37–42.
2. Guo, M.; Perez, C.; Wei, Y. Iron-binding properties of plant phenolics and cranberry's bio-effects. *R. Soc. Chem.* **2007**, 4951–4961.
3. Nelson, A. C. *Leninger: Principles of Biochemistry*, 4th ed.; Freeman and Company: New York, 2005.
4. Pellegrini, N. S. M.; Colombi, B. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *Am. Soc. Nutr. Sci.* **2003**, *133*, 2812–2819.
5. McGinnis, J. F.; Chen, J. P.; Patil, S.; Seal, S. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nat. Nanotechnol.* **2006**, *1* (2), 142–150.
6. Das, M. P. S.; Bhargava, N. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials* **2007**, *28*, 1918–1925.
7. Korsvik, C.; Patil, S.; Seal, S.; Self, W. T. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem. Commun.* **2007** (10), 1056–1058.
8. Self, W. T.; Heckert, E. G.; Karakoti, A. S.; Seal, S. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials* **2008**, *29* (18), 2705–2709.
9. Rotello, V. M.; Hong, R.; Han, G.; Fernandez, J. M.; Kim, B. J.; Forbes, N. S. Glutathione-mediated delivery and release using monolayer protected nanoparticle carriers. *J. Am. Chem. Soc.* **2006**, *128* (4), 1078–1079.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

10. Schubert, D.; Dargusch, R.; Raitano, J.; Chan, S. W. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem. Biophys. Res. Commun.* **2006**, *342* (1), 86–91.
11. Saikia, J. P.; Paul, S.; Konwar, B. K.; Samdarshi, S. K. Nickel oxide nanoparticles: A novel antioxidant. *Colloids Surf., B* **2010**, *78*, 146–148.
12. Miyamoto, Y.; Kim, J.; Takahashi, M.; Shimizu, T.; Shirasawa, T.; Kajita, M.; Kanayama, A. Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of *Caenorhabditis elegans*. *Mech. Ageing Dev.* **2008**, *129* (6), 322–331.
13. Aoshiba, K.; Onizawa, S.; Kajita, M.; Miyamoto, Y.; Nagai, A. Platinum nanoparticle antioxidants inhibit pulmonary inflammation in mice exposed to cigarette smoke. *Pulm. Pharmacol. Ther.* **2009**, *22* (4), 340–349.
14. Miyamoto, Y.; Watanabe, A.; Kajita, M.; Kim, J.; Kanayama, A.; Takahashi, K.; Mashino, T. In vitro free radical scavenging activity of platinum nanoparticles. *Nanotechnology* **2009**, *20* (45), 455105.
15. Barathmanikanth, S.; Kalishwaralal, K.; Sriram, M.; Pandian, S. R.; Youn, H. S.; Eom, S.; Gurunathan, S. Anti-oxidant effect of gold nanoparticles restrains hyperglycemic conditions in diabetic mice. *J. Nanobiotechnol.* **2010**, *8*, 16.
16. Esumi, K.; Takei, N.; Yoshimura, T. Antioxidant-potentiality of gold-chitosan nanocomposites. *Colloids Surf., B* **2003**, *32* (2), 117–123.
17. Paul, S. S. J. P.; Samdarshi, S. K. Investigation of antioxidant property of iron oxide particles by 1′-1 diphenylpicryl-hydrazyle (DPPH) method. *J. Magn. Magn. Mater.* **2009**, *321*, 3621–3623.
18. Garcia, H.; Martin, R.; Menchon, C.; Apostolova, N.; Victor, V. M.; Alvaro, M.; Herance, J. R. Nano-jewels in biology. Gold and platinum on diamond nanoparticles as antioxidant systems against cellular oxidative stress. *ACS Nano* **2010**, *4* (11), 6957–6965.
19. Pirmohamed, T.; Dowding, J. M.; Singh, S.; Wasserman, B.; Heckert, E.; Karakoti, A. S.; King, J. E. S.; Seal, S.; Self, W. T. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem. Commun.* **2010**, *46* (16), 2736–2738.
20. Paul, S.; Saikia, J. P.; Samdarshi, S. K.; Konwar, B. K. Investigation of antioxidant property of iron oxide particles by 1′-1 diphenylpicryl-hydrazyle (DPPH) method. *J. Magn. Magn. Mater.* **2009**, *321*, 3621–3623.
21. Cabelli, D. E.; Barnese, K.; Gralla, E. B.; Valentine, J. S. Manganous phosphate acts as a superoxide dismutase. *J. Am. Chem. Soc.* **2008**, *130,* (14), 4604−4606.
22. Nocera, D. G.; Yang, J. Y. Catalase and epoxidation activity of manganese salen complexes bearing two xanthene scaffolds. *J. Am. Chem. Soc.* **2007**, *129* (26), 8192–8198.
23. Schubert, D. D. R. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem. Biophys. Res. Commun.* **2006**, *342* (1), 86–91.
24. Estevez, A. Y.; Pritchard, S.; Harper, K.; Aston, J. W.; Lynch, A.; Lucky, J. J.; Ludington, J. S.; Chatani, P.; Mosenthal, W. P.; Leiter, J. C.; Andreescu, S.; Erlichman, J. S. Neuroprotective mechanisms of cerium oxide nanoparticles

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

in a mouse hippocampal brain slice model of ischemia. *Free Radical Biol. Med.* **2011**, *51*, 1155–1163.

25. Tarnuzzer, R. W.; Colon, J.; Patil, S.; Seal, S. Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage. *Nano Lett.* **2005**, *5* (12), 2573–2577.
26. Das, M.; Patil, S.; Bhargava, N.; Kang, J. F.; Riedel, L. M.; Seal, S.; Hickman, J. J. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials* **2007**, *28*, 1918–1925.
27. Silva, G. A. Nanomedicine: Seeing the benefits of ceria. *Nat. Nanotechnol.* **2006**, *1* (2), 92–94.
28. Baker, C. H.; Colon, J.; Hsieh, N.; Ferguson, A.; Kupelian, P.; Seal, S.; Jenkins, D. W. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. *Nanomed.: Nanotechnol., Biol. Med.* **2010**, *6* (5), 698–705.
29. Celardo, I.; De Nicola, M.; Mandoli, C.; Pedersen, J. Z.; Traversa, E.; Ghibelli, L. Ce(3+) ions determine redox-dependent anti-apoptotic effect of cerium oxide nanoparticles. *ACS Nano* **2011**, *5* (6), 4537–4549.
30. Karakoti, A. S.; Monteiro-Riviere, N. A.; Aggarwal, R.; Davis, J. P.; Narayan, R. J.; Self, W. T.; McGinnis, J.; Seal, S. Nanoceria as antioxidant: Synthesis and biomedical applications. *JOM* **2008**, *60* (3), 33–37.
31. Karakoti, A. S.; Singh, S.; Kumar, A.; Malinska, M.; Kuchibhatla, S. V. N. T.; Wozniak, K.; Self, W. T.; Seal, S., PEGylated nanoceria as radical scavenger with tunable redox chemistry. *J. Am. Chem. Soc.* **2009**, *131*, (40), 14144−14145.
32. Campbell, C. T.; Peden, C. H. F. Chemistry: Oxygen vacancies and catalysis on ceria surfaces. *Science* **2005**, *309* (5735), 713–714.
33. Seehra, M. S.; Dutta, P.; Pal, S.; Shi, Y.; Eyring, E. M.; Ernst, R. D. Concentration of Ce3+ and oxygen vacancies in cerium oxide nanoparticles. *Chem. Mater.* **2006**, *18* (21), 5144–5146.
34. Asati, A.; Santra, S.; Kaittanis, C.; Nath, S.; Perez, J. M. Oxidase-like activity of polymer-coated cerium oxide nanoparticles. *Angew. Chem., Int. Ed.* **2009**, *48* (13), 2308–2312.
35. Boaro, M.; Trovarelli, A.; Hwang, J. H.; Mason, T. O. Electrical and oxygen storage/release properties of nanocrystalline ceria-zirconia solid solutions. *Solid State Ionics* **2002**, *147* (1−2), 85–95.
36. Schalow, T.; Laurin, M.; Brandt, B.; Schauermann, S.; Guimond, S.; Kuhlenbeck, H.; Starr, D. E.; Shaikhutdinov, S. K.; Libuda, J.; Freund, H. J. Oxygen storage at the metal/oxide interface of catalyst nanoparticles. *Angew. Chem., Int. Ed.* **2005**, *44* (46), 7601–7605.
37. Trovarelli, A. Structural and oxygen storage/release properties of CeO2-based solid solutions. *Comments Inorg. Chem.* **1999**, *20* (4−6), 263–284.
38. Tsai, Y. Y.; Oca-Cossio, J.; Lin, S. M.; Woan, K.; Yu, P. C.; Sigmund, W. Reactive oxygen species scavenging properties of ZrO2-CeO2 solid solution nanoparticles. *Nanomedicine* **2008**, *3* (5), 637–645.

39. Seal, S.; Patil, S.; Sandberg, A.; Heckert, E.; Self, W. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials* **2007**, *28* (31), 4600–4607.
40. Ivanov, V. K.; Shcherbakov, A. B.; Usatenko, A. V. Structure-sensitive properties and biomedical applications of nanodispersed cerium dioxide. *Russ. Chem. Rev.* **2009**, *78* (9), 855–871.
41. Schubert, D. D. R. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem. Biophys. Res. Commun.* **2006**, *342* (1), 86–91.
42. Saikia, J. P.; Paul, S.; Konwar, B. K.; Samdarshi, S. K. Ultrasonication: Enhances the antioxidant activity of metal oxide nanoparticles. *Colloids Surf., B* **2010**, *79* (2), 521–523.
43. Pirmohamed, T.; Dowding, J. M.; Singh, S.; Wasserman, B.; Heckert, E.; Karakoti, A. S.; King, J. E.; Seal, S.; Self, W. T. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem. Commun. (Cambridge, U. K.)* **2010**, *46* (16), 2736–2738.
44. Yin, H. Quest for better antioxidants: A commentary on "Enhanced radical-scavenging activity by antioxidant-functionalized gold nanoparticles: A novel inspiration for development of new artificial antioxidant". *Free Radical Biol. Med.* **2007**, *43* (9), 1229–1230.
45. Liu, Y.; Nie, Z.; Liu, K. J.; Zhong, C. J.; Wang, L. F.; Yang, Y.; Tian, Q. Enhanced radical scavenging activity by antioxidant-functionalized gold nanoparticles: A novel inspiration for development of new artificial antioxidants. *Free Radical Biol. Med.* **2007**, *43* (9), 1243–1254.
46. Guo, M. L.; Perez, C.; Wei, Y. B.; Rapoza, E.; Su, G.; Bou-Abdallah, F.; Chasteen, N. D. Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton Trans.* **2007** (43), 4951–4961.
47. Yadav, S. C.; Kumari, A.; Yadav, S. K.; Pakade, Y. B.; Singh, B. Development of biodegradable nanoparticles for delivery of quercetin. *Colloids Surf., B* **2010**, *80* (2), 184–192.
48. Wu, T. H.; Yen, F. L.; Lin, L. T.; Tsai, T. R.; Lin, C. C.; Cham, T. M. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *Int. J. Pharm.* **2008**, *346* (1−2), 160–168.
49. Lee, H. G.; Jang, K. I. Stability of chitosan nanoparticles for L-ascorbic acid during heat treatment in aqueous solution. *J. Agric. Food Chem.* **2008**, *56* (6), 1936–1941.
50. Lin, C. C.; Yen, F. L.; Wu, T. H.; Tzeng, C. W.; Lin, L. T. Curcumin nanoparticles improve the physicochemical properties of curcumin and effectively enhance its antioxidant and antihepatoma activities. *J. Agric. Food Chem.* **2010**, *58* (12), 7376–7382.
51. Chorny, M.; Hood, E.; Levy, R. J.; Muzykantov, V. R. Endothelial delivery of antioxidant enzymes loaded into non-polymeric magnetic nanoparticles. *J. Controlled Release* **2010**, *146* (1), 144–151.
52. Gupta, A. K.; Gupta, M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* **2005**, *26* (18), 3995–4021.
53. Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Elst, L. V.; Muller, R. N. Magnetic iron oxide nanoparticles: Synthesis, stabilization, vectorization,

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

physicochemical characterizations, and biological applications. *Chem. Rev.* **2008**, *108* (6), 2064–2110.

54. Gao, J. H.; Gu, H. W.; Xu, B. Multifunctional magnetic nanoparticles: Design, synthesis, and biomedical applications. *Acc. Chem. Res.* **2009**, *42* (8), 1097–1107.
55. Huang, D. J.; Ou, B. X.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53* (6), 1841–1856.
56. Covaliu, C. I.; Matei, C.; Litescu, S.; Eremia, S. A. M.; Stanica, N.; Diamandescu, L.; Ianculescu, A.; Jitaru, I.; Berger, D. Radical scavenger properties of oxide nanoparticles stabilized with biopolymer matrix. *Mater. Plast. (Bucharest, Rom.)* **2010**, *47* (1), 5–10.
57. Zhang, Z. Y.; Zhang, H. Z.; H., F.; He, X. A.; Zhang, P.; Li, Y. Y.; Ma, Y. H.; Kuang, Y. S.; Zhao, Y. L.; Chai, Z. F. Nano-CeO(2) exhibits adverse effects at environmental relevant concentrations. *Environ. Sci. Technol.* **2011**, *45* (8), 3725–3730.
58. Richman, E. K.; Hutchison, J. E. The nanomaterial characterization bottleneck. *ACS Nano* **2009**, *3* (9), 2441–2446.
59. Hardman, R. A toxicologic review of quantum dots: Toxicity depends on physicochemical and environmental factors. *Environ. Health Perspect.* **2006**, *114* (2), 165–172.
60. Fahmy, B.; Cormier, S. A. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. *Toxicol. in Vitro* **2009**, *23* (7), 1365–1371.
61. Limbach, L. K.; Li, Y. C.; Grass, R. N.; Brunner, T. J.; Hintermann, M. A.; Muller, M.; Gunther, D.; Stark, W. J. Oxide nanoparticle uptake in human lung fibroblasts: Effects of particle size, agglomeration, and diffusion at low concentrations. *Environ. Sci. Technol.* **2005**, *39* (23), 9370–9376.
62. Ge, Y. Q.; Zhang, Y.; Xia, J. G.; Ma, M.; He, S. Y.; Nie, F.; Gu, N. Effect of surface charge and agglomerate degree of magnetic iron oxide nanoparticles on KB cellular uptake in vitro. *Colloids Surf., B* **2009**, *73* (2), 294–301.
63. Karakoti, A. S.; Hench, L. L.; Seal, S. The potential toxicity of nanomaterials: The role of surfaces. *JOM* **2006**, *58* (7), 77–82.
64. Faraji, A. H.; Wipf, P. Nanoparticles in cellular drug delivery. *Bioorg. Med. Chem.* **2009**, *17* (8), 2950–62.
65. Liong, M.; Lu, J.; Kovochich, M.; Xia, T.; Ruehm, S. G.; Nel, A. E.; Tamanoi, F.; Zink, J. I. Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. *ACS Nano* **2008**, *2* (5), 889–896.
66. Pierscionek, B. K.; Li, Y. B.; Yasseen, A. A.; Colhoun, L. M.; Schachar, R. A.; Chen, W. Nanoceria have no genotoxic effect on human lens epithelial cells. *Nanotechnology* **2010**, *21* (3), 035102.
67. Kasemo, B.; Lausmaa, J. Material-tissue interfaces: The role of surface-properties and processes. *Environ. Health Perspect.* **1994**, *102*, 41–45.
68. Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. Understanding biophysicochemical interactions at the nano-bio interface. *Nat. Mater.* **2009**, *8* (7), 543–557.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

69. Self, W. T.; Heckert, E. G.; Seal, S. Fenton-like reaction catalyzed by the rare earth inner transition metal cerium. *Environ. Sci. Technol.* **2008**, *42* (13), 5014–5019.
70. Andreescu, S.; Ornatska, M.; Sharpe, E.; Andreescu, D. Paper bioassay based on ceria nanoparticles as colorimetric probes. *Anal. Chem.* **2011**, *83* (11), 4273–4280.
71. Hong, F. S.; Ma, L. L.; Liu, J.; Li, N.; Wang, J.; Duan, Y. M.; Yan, J. Y.; Liu, H. T.; Wang, H. Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO(2) delivered to the abdominal cavity. *Biomaterials* **2010**, *31* (1), 99–105.
72. Hritcu, L.; Stefan, M.; Ursu, L.; Neagu, A.; Mihasan, M.; Tartau, L.; Melnig, V. Exposure to silver nanoparticles induces oxidative stress and memory deficits in laboratory rats. *Cent. Eur. J. Biol.* **2011**, *6* (4), 497–509.
73. Simon, U.; Pan, Y.; Leifert, A.; Ruau, D.; Neuss, S.; Bornemann, J.; Schmid, G.; Brandau, W.; Jahnen-Dechent, W. Gold nanoparticles of diameter 1.4 nm trigger necrosis by oxidative stress and mitochondrial damage. *Small* **2009**, *5* (18), 2067–2076.
74. Li, J. J.; Hartono, D.; Ong, C.-N.; Bay, B.-H.; Yung, L.-Y. L. Autophagy and oxidative stress associated with gold nanoparticles. *Biomaterials* **2010**, *31* (23), 5996–6003.
75. Tedesco, S.; Doyle, H.; Blasco, J.; Redmond, G.; Sheehan, D. Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis*. *Aquat. Toxicol.* **2010**, *100*, 178−186.
76. Kumar, M. N. V. R.; Ratnam, D. V.; Ankola, D. D.; Bhardwaj, V.; Sahana, D. K. Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. *J. Controlled Release* **2006**, *113* (3), 189–207.
77. Hickman, J. J.; Das, M.; Patil, S.; Bhargava, N.; Kang, J. F.; Riedel, L.; Seal, S. Auto-catalytic Ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Tissue Eng.* **2007**, *13* (4), 873–874.
78. Elswaifi, S. F.; Palmieri, J. R.; Hockey, K. S.; Rzigalinski, B. A. Antioxidant nanoparticles for control of infectious disease. *Infect. Disord.: Drug Targets* **2009**, *9* (4), 445–52.
79. Niu, J.; Azfer, A.; Rogers, L. M.; Wang, X.; Kolattukudy, P. E. Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy. *Cardiovasc. Res.* **2007**, *73* (3), 549–59.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch008

Chapter 9

Cerium Oxide Nanoparticles for the Treatment of Neurological Oxidative Stress Diseases

A. Y. Estevez*,[1,2] and J. S. Erlichman[1]

[1]Biology Department, St. Lawrence University,
Canton, New York 13617
[2]Psychology Department, St. Lawrence University,
Canton, New York 13617
***E-mail: aestevez@stlawu.edu**

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Due to their potent free radical scavenging properties, cerium oxide (CeO_2) nanoparticles (nanoceria) are widely used as catalysts in industrial applications. However, given the prominent role of free radicals in the pathology of many human diseases, these nanoparticles are now being explored for therapeutic purposes in the treatment of oxidative stress disorders. This review highlights the various sources of free radicals in neurological oxidative stress diseases and the use of antioxidants to mitigate free radical damage. In particular, recent studies exploring the use of CeO_2 nanoparticles as therapeutic antioxidants are discussed.

Oxidative Stress Biology

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are potent oxidizing and nitrating agents that include superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$). Although most ROS/RNS are also considered to be free radicals (molecules with unpaired electrons in the outer orbital shell), some are not (e.g., H_2O_2 and $ONOO^-$). ROS and RNS are produced under both physiological and pathophysiological conditions and can be derived from a multitude of cellular sources (reviewed in (*1–3*)). For example, mitochondria produce several ROS

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

as a byproduct of cellular respiration and immune cells generate $O_2^{\bullet-}$ and NO as defense mechanisms against infectious organisms (*4*, *5*). Physiological levels of $O_2^{\bullet-}$ and NO have been shown to play a role in signal transduction in the nervous and cardiovascular systems (*3*, *6–10*). Peroxisomes, organelles important in the oxidation of fatty acids as well as toxic substances (e.g., ethanol) contain several enzymes that can produce reactive oxygen species including H_2O_2 (reviewed in (*11*)). In addition to biological sources, we are exposed to free radicals from environmental sources including cigarette smoke, car exhaust and ultraviolet radiation from the sun.

Because of their high reactivity and potential for inducing damage, cells and organisms possess endogenous systems to neutralize free radicals and reactive oxygen species. These include enzyme antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase and non-enzymes like glutathione. SODs catalyze the dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2 using metal prosthetic groups (Cu/Zn or Mn, in mammals) (*12*, *13*) and are expressed in the mitochondria, cytosol and extracellular space. Thus, even though a certain amount of $O_2^{\bullet-}$ might ‘leak’ from the electron transport chain, mitochondrial SOD limits the amount of oxidative damage that might otherwise result. Catalases are ubiquitously expressed and catalyze the conversion of H_2O_2 into O_2 and H_2O (*14*, *15*). Finally, glutathione peroxidases catalyze the conversion of hydrogen and lipid peroxides into less harmful metabolites (*16*, *17*). In addition to these, exogenous antioxidants such as those obtained from dietery sources like ascorbic acid (Vitamin A), tocopherol (Vitamin E), carotenoids and flavanoids also help limit the effects of ROS and free radicals (*18*, *19*).

When the level of free radicals exceeds the ability of antioxidant systems to inactivate or neutralize them, the result is a state of oxidative stress. Oxidative stress plays a role in aging as well as a variety of human disease states. The involvement of free radicals in the normal aging process was an idea initially put forth by Harman in 1956 (*20*) as the ‘free radical theory of aging’. Although the idea that free radicals are the cause of aging has fallen out of disfavor (e.g., (*21*, *22*)), there is still a vast amount of data supporting the notion that oxidants nonetheless still contribute to the aging process (*23*). For example, long term exposure to free radical scavengers/antioxidants is neuroprotective and increases life-span in a wide variety of model systems (*24–27*). The increase in oxidative stress with aging can be attributed to an increase in oxidant load, decrease in antioxidant capacity and decreased ability to repair oxidative damage over time (*28–30*).

The brain is particularly susceptible to oxidative stress due to high oxygen utilization, low levels of endogenous antioxidant systems and high levels of polyunsaturated fatty acids (PUFAs) which are subject to lipid peroxidation (*31*). Not surprisingly then, oxidative stress plays a prominent role in the pathology of a plethora of neurological disorders including cerebral ischemia, Alzheimer’s disease, Parkinson’s disease and Amyotrophic lateral sclerosis (ALS), to just name a few. As this has been thoroughly reviewed elsewhere (*32–37*), in the following sections we will briefly highlight the etiology of each disorder with a focus on the role of ROS/RNS in disease pathogenesis.

Cerebral Ischemia or Stroke

Cerebral ischemia or stroke, caused by a reduction of blood flow to the brain due to either a clot or hemorrhage, is the third leading cause of death and the leading cause of long term disability in the United States (*38*). The lack of energy production that occurs as a result of the reduction in glucose and oxygen delivery to brain cells rapidly initiates a of sequence of biochemical reactions, termed the ischemic cascade, that can lead to cellular demise (Figure 1). Briefly, bioenergetic failure leads to a disruption in ionic homeostasis which consequently induces excitotoxicity, oxidative stress, inflammation, blood-brain barrier dysfunction and ultimately, cell death (reviewed in (*39*)). It is estimated that every minute of a stroke results in the loss of 1. 9 million neurons and 14 billion synapses, equivalent to 3.1 weeks of aging (*40*).

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

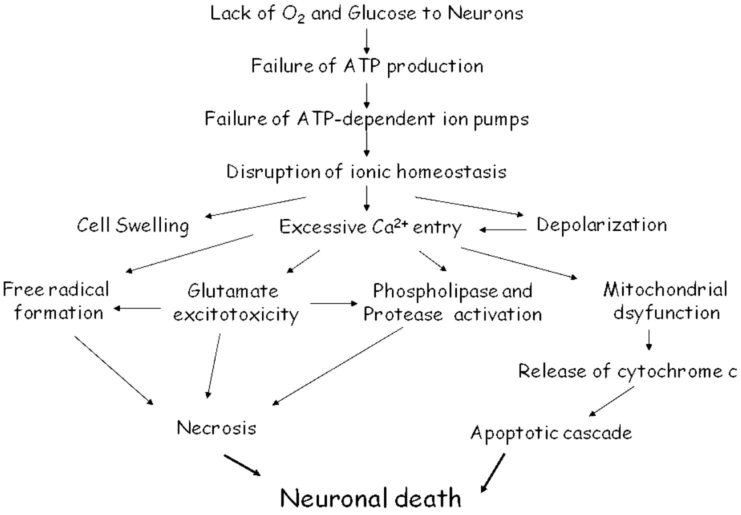

Lack of O2 and Glucose to Neurons
Failure of ATP production
Failure of ATP-dependent ion pumps
Disruption of ionic homeostasis
Cell Swelling
Excessive Ca2+ entry
Depolarization
Free radical formation
Glutamate excitotoxicity
Phospholipase and Protease activation
Mitochondrial dsyfunction
Release of cytochrome c
Necrosis
Apoptotic cascade
Neuronal death

Figure 1. Schematic diagram of the ischemic cascade.

Oxidative Stress and Stroke

The production of free radicals and ROS is associated with many of the pathways involved in ischemic cell death (Figure 2). Disruption of the electron transport chain leads to the production of $O_2^{\bullet-}$ and H_2O_2 by the mitochondria (*41–43*). Glutamate excitotoxicity leads to the over-activation of NMDA receptors and causes a rise in intracellular calcium that activates enzymes such as nitric oxide synthase (NOS) and phospholipase A2 which generate NO and arachidonic acid, respectively. As a free radical, NO can have damaging effects in and of itself, but can also react with $O_2^{\bullet-}$ (from mitochondrial leak) to generate the highly toxic $ONOO^-$, which acts as both an oxidant and a nitrating agent to cause cellular damage (*44–47*). Although $ONOO^-$ is labile, it has a long enough half-life to diffuse in tissues and react with CO_2 to form other reactive intermediates that lead to sustained protein modifications such as the formation of 3-nitrotyrosine (3-NT) (*48–50*). The metabolism of arachidnoic acid via cyclooxygenase and lipoxygenase pathways also contributes to the generation of ROS and lipid peroxides (*51*, *52*). Xanthine oxidase and NADPH oxidase have also been shown to be prominent sources of ROS during the reperfusion period (*33*, *42*, *53*). Finally, the activation of microglia, the brain's resident immune cells, generates an inflammatory response which includes the production of ROS (*54–56*). In summary, the ROS/RNS derived from various sources accumulate during both the ischemia and the reperfusion period (e.g., (*57*, *58*)) and this oxidative stress can ultimately contribute to cell death via necrotic or apoptotic pathways (*33*, *36*, *59–61*).

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

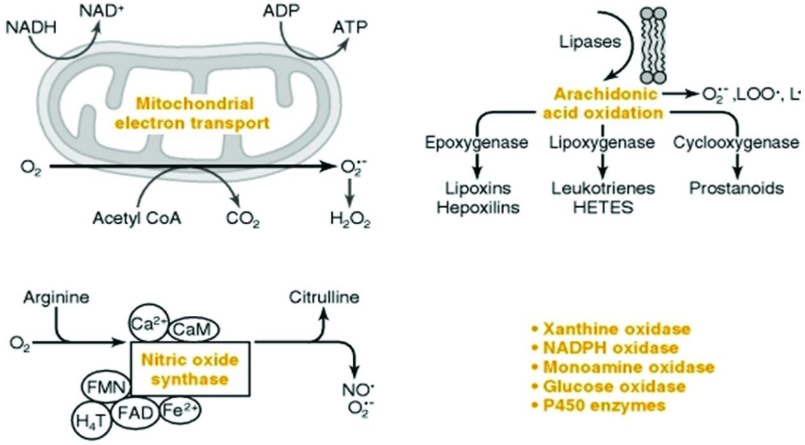

NADH
NAD⁺
ADP
ATP
Mitochondrial electron transport
O_2
$O_2^{\cdot -}$
Acetyl CoA
CO_2
H_2O_2
Lipases
Arachidonic acid oxidation
$O_2^{\cdot -}$,LOO·, L·
Epoxygenase
Lipoxygenase
Cyclooxygenase
Lipoxins
Hepoxilins
Leukotrienes
HETES
Prostanoids
Arginine
O_2
Ca^{2+}
CaM
Nitric oxide synthase
FMN
H_4T
FAD
Fe^{2+}
Citrulline
NO·
$O_2^{\cdot -}$
• Xanthine oxidase
• NADPH oxidase
• Monoamine oxidase
• Glucose oxidase
• P450 enzymes

Figure 2. Sources of ROS during ischemia. Reproduced with permission from reference (62). Copyright 1999 American Society for Neurochemistry. (see color insert)

The only currently approved treatment for stroke involves the use of thrombolytic agents (a.k.a. clot-busting drugs) such as recombinant tissue plasminogen activator (rt-PA or Alteplase) to restore blood flow. However, rt-PA has limited use because it is only effective when administered within a short time-frame after stroke onset and it is not indicated for use in hemorrhagic strokes (*63*). The are currently no approved neuroprotective therapies – those that are aimed at minimizing neuronal death – for the treatment of stroke.

Parkinson's Disease (PD)

Parkinson's Disease, the second most common neurodegenerative disorder, is caused by the death of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. As these cells are important in the basal ganglia circuit regulating

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

movement, symptoms of the disease (which do not become apparent up until about 50-80% of the cells have died) include tremor at rest, difficulty initiating movements and slow movements (bradykinesia). A key histopathological finding is the presence of proteinaceous cytoplasmic inclusions termed Lewy bodies which accumulate gradually throughout the disease and are comprised of several proteins including α-synuclein, ubiquitin and parkin (*64*). Although the cause of dopaminergic neurodegeneration is unknown, age, environmental factors and genetics have all been shown increase the risk of developing PD. In addition, cell-specific factors such the normal metabolism of dopamine by monoamine oxidase B (MAOB) (*65*, *66*) and increased calcium channel activity (*67*) have been proposed to underly the susceptibility of substantia nigra neurons to oxidative stress and neurodegeneration.

Oxidative Stress and PD

Although 5% of the cases of PD are familial (genetic) due to mutations in genes encoding proteins such as α-synuclein and parkin, the vast majority of PD cases are classified as sporadic (unknown cause). Many hypotheses regarding the etiology of sporadic PD revolve around mitochondrial dysfunction and oxidative stress (*30*, *34*, *35*, *68*). In support of this notion, post-mortem analysis of the brains of PD patients identified reduced levels of NADH-ubiquinone reductase (mitochondrial Complex I) in the substantia nigra (*69*, *70*). Other anatomical and functional data further support the role of protein oxidation and nitration in PD (*34*, *71*). In addition, the key animal models of PD involve the use of toxins that selectively destroy catecholamine systems (dopaminergic and noreadrinergic) via oxidative stress. The two most commonly used toxins are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (*72*). The mechanism of toxicity of 6-OHDA involves oxidative stress via multiple mechanisms including disruption of mitochondrial Complex I, increased formation of superoxide, inhibition of endogenous antioxidant systems such as SOD and glutathione (*73*, *74*) as well as the normal metabolism of 6-OHDA by MAOB. MPTP, discovered accidentally as a dopminergic neurotoxin in the early 1980's when a group of young addicts developed early parkinsonism after intravenous administration of synthetic heroin (*75–77*), gets taken up into neurons by monoamine (dopamine and norepinephrine) transporters (similar to 6-OHDA) and gets metabolized into 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ has been shown to disrupt Complex I.

Currently approved pharmacological treatments for PD are aimed are reducing the symptoms of the disease. For example, the dopamine precursor levodopa (L-dopa) or dopamine receptor agonists are given with the aim of enhancing dopaminergic transmission in the remaining substantia nigra neurons. Although L-dopa is considered the 'gold standard' for the treatment of PD, effectiveness typically wanes after 5 years and the side effects, such as dyskinesia (jerky, involuntary movements) and psychosis (*78*) can be debilitating. As with stroke, there are currently no available therapies aimed at halting dopamine neuron degeneration.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Alzheimer's Disease (AD)

Alzheimer's disease is the most common neurodegenerative disorder and the primary cause of dementia in the elderly. Dementia is defined as a decline of memory and at least one other cognitive function such as attention, judgement, or decision making. AD involves the progressive degeneration of neurons beginning at the entorhinal cortex and hippocampal cornu ammonis cell layer 1 (CA1) and encompassing the entirety of the hippocampus then the rest of the neocortex at later stages (*79*). Because of the progressive nature of the disease, early symptoms involve subtle loss of memory whereas later symptoms involve profound memory loss, confusion, spatial disorientation and alterations in mood and behavior. During the very late stages of the disease, patients lose their ability to speak, move and care for themselves.

Age is considered the primary risk factor for AD with the rate of diagnosis increasing as the individual ages beyond 65 years old (*80*, *81*). In fact, nearly half of all individuals over the age of 85 are diagnosed with probable AD (AD diagnosis must be confirmed post-mortem) (*81*). Genetics plays a role in ~5% of AD cases with mutations in amyloid precursor protein (APP) and the presenelins 1 and 2 (PSEN1 and PSEN2) contributing to early onset or familial AD (diagnosed prior to age 65). However, the vast majority of AD cases are of late onset and the primary risk factors are age (as mentioned above) and the presence of the ε4 allele of apolipoprotein (APOε4), a protein normally involved in the maintenance and repair of neurons (*82*).

The two hallmark abnormalities found in an AD brain are extracellular plaques and intracellular neurofibrillary tangles. The plaques are comprised of β-amyloid derived from the processing of amyloid precursor protein (APP) into smaller peptitdes containing 39 to 42 amino acid residues. $A\beta_{1\text{-}42}$ plays a critical role in disease pathogenesis due to it's propensity to aggregate and form neuritic plaques (*83*, *84*). The key components of neurofibrillary tangles are paired helical fragments comprised of tau protein. Tau proteins normally play an important role in the stabilization of microtubules (*85*), organelles important in the maintenance of neuronal structure. Hyperphosphorylation of tau (*86*) due to aberrant signal transduction in the AD brain, particularly an imbalance of kinase and phosphatase activity (*87*), leads to the destabilization of microtubules and subsequent formation of neurofibrillary tangles.

Oxidative Stress and AD

Redox status plays a critical role in AD pathology. Increased lipid peroxidation, nucleic acid oxidation and reduced antioxidant activity (e.g., SOD) have all been reported in AD brains (*88–92*). $A\beta_{1\text{-}42}$ has been shown to cause an increase in H_2O_2 via Fenton chemistry (*93*) as well as generate other reactive oxygen species and peptides (*84*, *94*, *95*). Activated microglia, which surround the neuritic plaques, release NO and $O_2^{\bullet-}$ in an effort to clear them (*56*, *96*). As is the case with many other neurodegenerative diseases, impaired mitochondrial function has been proposed to play a key role in AD etiology (*97–102*). In particular, accumulation of $A\beta_{1\text{-}42}$ in the mitochondria of transgenic mouse model of AD had been implicated in ROS generation (*103*). There is, however, still debate as to whether oxidative imbalance is an early event (*104*, *105*) as opposed to a final common pathway to cell death (*101*). The recent finding that mitochondrial dysfunction tends to precede the formation of plaques suggests that oxidative stress is an early event (*106*).

Two categories of drugs are currently approved for the treatment of AD (*107*). Cholinesterase inhibitors such as donepezil and tacrine are used to treat early symptoms of cognitive decline by enhancing cholinergic transmission. For later stages memantine, a glutamate NMDA receptor antagonist, is prescribed to slow or limit neuronal death due to excitotoxicity. However, there are currently no drugs approved that halt the disease. Progress in developing such a neuroprotective agent has been stymied by some of the same issues that have hampered development of stroke therapeutics. In particular, the effectiveness of compounds in animal models of AD has not translated into effectiveness at the clinical trials (*108*).

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Multiple Sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) in which the destruction of myelin and oligodendrocytes by autoreactive T and B cells causes focal demyelinated plaques and nerve loss throughout the brain and the spinal cord. Typical early symptoms include numbness, tingling, limb weakness and blurred vision. As the disease progresses fatigue, dizziness, gait disturbances, vision problems and difficulty with speech and swallowing are observed (*109*). Four different disease courses have been identified. Most cases (~85%) of MS are relapsing-remitting in which neurological attacks or flare-ups are followed by complete recovery. Secondary progressive MS is diagnosed when an initial relapsing-remitting course (~38%) turns into one without remissions. A small percentage of individuals are diagnosed with primary progressive MS in which the symptoms never wane from the onset of the disease. Finally, the most rare form of MS is progressive-relapsing in which the symptoms continually get worse from the onset, with defined instances of flare-ups along the way. The cause of MS is unknown. However, genetics, environment and host factors have all been proposed to play a role in the etiology of the disease (*110–112*).

Oxidative Stress and MS

Experimental autoimmune encephalomyelitis (EAE) is an experimentally induced inflammatory central nervous system demyelinating disease that mimics many aspects of relapsing multiple sclerosis (MS) in humans (*113*). EAE involves ROS-mediated neuronal damage and destruction (*114–116*). Oxidative damage is preceded by infiltration of immune cells typically kept apart from the central nervous system (CNS) by the blood-brain barrier (BBB) (*117*). Infiltrating TH1 cells secrete interferon-gamma (IFN-γ), which activates macrophages that generate an inflammatory milieu via the production and secretion of the inflammatory cytokines IL-1 (*118*, *119*) and TNF-α (*120*) as well as ROS (*116*).

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Interest has recently focused on TH17 cells in EAE, and IL-17 induces the production of ROS in endothelial cells of the BBB (*121*) which disrupts tight junctions between these cells and increases permeability of the BBB (*122*). Thus, whether the polarity of the TH response in EAE is TH1 or TH17, ROS are involved in generating disease pathology of demyelination, probably by virtue of the production of NO and its derivative, $ONOO^-$ (*123*). iNOS, one of the key enzymes involved in synthesis of NO, is upregulated in acute inflammatory MS lesions (*124–126*). Elevated levels of NO can have a detrimental effect on axonal survival (*126*) by modifying the action of ion channels, transporters, and glycolytic enzymes (*127–129*). NO and peroxynitrite also inhibit mitochondrial respiration (*130*) and limit the axon's ability to generate ATP. Although peroxynitrite is both a potent oxidant and nitrosylates protein, NO may serve multiple regulatory roles. For example, animals with absent or disrupted NOS2 (*131*) or iNOS (*132*) espression display more severe EAE symptoms than wild-type animals. NO may dampen TH cell proliferation and disrupt targeted migration of inflammatory cells into the CNS (*133*). Thus, NO may oppose the function of the autoreactive TH cells that initiate damage, while at the same time eliciting its own neurotoxic effects. This paradox makes it a challenge to identify therapeutic agents that reduce NO levels sufficiently to mitigate direct CNS damage, but still leave intact the beneficial aspects of NO-mediated immunomodulation.

Although EAE in mice may not reproduce all of the pathogenic mechanisms of the human disease, the validity of this model has been repeatedly confirmed. Most importantly, the study of EAE has led to the development of three currently available MS therapies. These, not surprisingly because of disease etiology, all function as immune modulators: Avonex and Betaferon, both of which are forms of beta interferon; and glatiramer acetate (Copaxone), which is an immune modulator that may shift T helper (TH) cells from an inflammatory TH1 to a TH2 phenotype or act as a decoy for the immune (*134*, *135*). However because of the established role of oxidative stress in MS, drugs that target ROS production might be useful as a co-therapy. To that end, there are three promising drugs on the horizon: GEMSP, nanoliposomes containing tempamine, and edavarone (also used in stroke), all of which are antioxidants (*136–138*).

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), often referred to as "Lou Gehrig's Disease," is a progressive, fatal, neurodegenerative disease that affects motor neurons in the brain and the spinal cord (*139*, *140*). As motor neurons degenerate, they can no longer send impulses to the muscle fibers that normally result in muscle movement. As such, early symptoms of ALS often include increasing muscle weakness, especially involving the arms and legs, speech, swallowing or breathing. When the motor neurons die, the ability of the brain to initiate and control muscle movement is lost. With voluntary muscle action progressively affected, patients in the later stages of the disease may become totally paralyzed with death occurring due to respiratory failure, usually between 2 and 5 years post-diagnosis (*141*).

Approximatley 90-95% of the cases of ALS are sporadic (SALS; unknown cause) with the remaining 5-10% classified as familial (FALS; genetic). The majority of the familial cases, ~20%, are due to mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene (*142*, *143*). The mechanism of mutant SOD1 toxicity seems to be due to a gain of toxic function that leads to catalysis of aberrant oxidative reactions (*143–145*).

Oxidative Stress and ALS

There is an overwhelming amount of data supporting the involvement of oxidative stress in ALS pathology. For example, post-mortem analysis of brain tissue samples show increased levels of protein and DNA oxidation in the motor cortex of patients with sporadic ALS (*146*). In addition, spinal cords of both FALS and SALS patients show increased levels of lipid, protein and DNA oxidation (*146–148*) as well as increased 3-nitrotyrosine immunoreactivity, a marker of $ONOO^-$ induced damage. Finally, all of these markers of oxidative stress are elevated in serum and cerebrospinal fluid samples of living ALS patients (*149*, *150*). The question that has not been resolved, however, is whether oxidative stress is a cause or a result of the underlying ALS etiology.

Laboratory models of ALS help researchers understand the basic process of the disease, with the goal of developing new therapies. The mainstay has been a mouse that bears the mutated human gene associated with familial ALS. A mutation of the SOD1 gene can produce many aspects of ALS. The mouse bearing the human gene for mutant SOD1 was the first lab model clearly linked to ALS based on a known cause of the disease. The ALS mouse has helped with the testing of drugs that might be useful for treating the disease. However, there is still no cure or treatment today that halts or reverses ALS (*151*). There is one FDA approved drug, riluzole, that modestly slows the progression of ALS (*152*). Riluzole interferes with glutamatergic neurotransmission and might therefore mitigate downstream ROS production by reducing excitotoxicity.

Cerium Oxide Nanoparticles as a Next Generation Antioxidant Therapy for Neurodegenerative Diseases

As is evident from the above survey, ROS and mitochondrial dysfunction play a key role in the pathology of many neurodegenerative diseases. Despite this overwhelming evidence of oxidative injury and the efficacy of antixodiants in pre-clinical animal models, human studies of antioxidant therapies have had mixed results (Table I). The case of antioxidants for stroke provides an illustrative example. Although several antioxidant compounds have made it to clinical trials, the recent high-profile failure of the nitrone spin trap agent NXY-059 has called into question whether pursuing neuroprotection strategies is worth it (*153*). There was a lot of initial hope with NXY-059 because in preclinical studies it demonstrated consistent protective effects in stroke models, the antixodant mechanism of action (spin trap) was slightly different than most other antixodiants (i.e., no generation of other free radicals in the process of

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

scavenging ROS) and it's clinical evaluation followed the recommendations made by the Stroke Therapy Academic Industry Roundtable (STAIR) (*154*). In the initial trial, SAINT I (Stroke-Acute Ischemic NXY-059 Treatment) (*155*), NXY-059 improved the primary output measure – disability at 90 days. However, Astra-Zeneca abrubtly halted development of NXY-059 when the results of the SAINTS II trial, a repeat study with greater sample size, showed lack of efficacy (*156*). Some of the reasons cited in retrospect for the failure of NXY-059 include lack of rigor in the design and interpretation of results from pre-clinical studies (i.e., it should probably not have made it to clinical trials) to the rationale for the enrollment time window used in the actual clinical studies (*157*, *158*).

The lack of success with antioxidant therapies in general may be due to multiple factors (*159*, *160*): an inability to achieve satisfactory levels of antioxidants at the site of injury (this is especially true of the CNS); the agents are consumed at a rate greater than their entry rate so that sufficient antioxidant levels cannot be sustained; the antioxidants tried so far have not actually been that potent; or the antioxidant may not neutralize the principal ROS involved in the pathology. Additionally, in many cases the production of free radicals occurs rapidly and early in the disease process, and administration of antioxidant agents after the initial injury is ineffective.

Despite this seemingly grim outlook, there are currently some promising antioxidant prospects both at the clinical and pre-clinical stages. For example, Edaravone is a compound that was approved in 2001 for the treatment of acute ischemic stroke in Japan (*161*). It crosses the blood brain barrier and aside from its antioxidant properties has other targets in the ischemic cascade, such as eNOS, which might play a role in it's therapeutic effectiveness (*162–164*). In terms of agents in pre-clinical phases, the therapeutic potential of small molecule catalytic antioxidants is becoming apparent (*151*, *165–167*). Catalytic antioxidants can neutralize ROS at a rate approaching or exceeding that of SOD, providing an advantage over typical free radical scavengers (like Vitamin E and others) that neutralize at a 1:1 stoichiometric ratio. Of these compounds, the manganese porphyrin AEOL 11207 has been shown to penetrate the BBB and display neuroprotective effectiveness in the mouse MPTP model of PD after either oral or subcutatneous administration (*168*). Cerium oxide (CeO_2) nanoparticles, or nanoceria, have the added advantage of being regenerative in nature. Thus, nanoceria have great potential for the treatment oxidative neurological disorders that overcome many of the deficiencies of previous therapies and this will be discussed in more detail below.

Cerium is a rare earth metal belonging to the lanthanide series of the periodic table. When combined with oxygen in a nanoparticle formulation, cerium oxide adopts a fluorite crystalline structure that has unique antioxidant properties (*166*, *198*) derived from the kinetics and thermodynamics of the redox processes on the nanoparticle surface. Cerium can reversibly bind oxygen and shift between Ce^{4+} and Ce^{3+} states under oxidizing and reducing conditions. The loss of oxygen and the reduction of Ce^{4+} to Ce^{3+} are accompanied by the creation of oxygen vacancies in the nanoparticle lattice (*199*, *200*). Nanoceria have been demonstrated to display both superoxide dismutase- and catalase-mimetic activity (*167*, *198*) and it is this dual capability that, according to a model proposed by recently by Celardo et al., endows nanoceria the ability to regenerate its antioxidant activity (*201*) . The model involves Ce^{3+} nanoceria reducing $O_2^{\bullet -}$ to form H_2O_2 and Ce^{4+}. The H_2O_2 formed can then react with Ce^{4+} to regenerate Ce^{3+} and form O_2 (Figures 3 and 4).

Table I. Efficacy of some selected antioxidants in human studies

Neurodegenerative Disease	***Antioxidant***	***Mechanism of Action***	***Outcome***	***Reference***
Stroke	NXY-059	free radical spin trapping agent	Improved primary output measure (disability at 90 days) but did not improve neurological outcome	SAINT I (*155*)
			Lack of efficacy	SAINT II (*156*, *169*)
	Edaravone	free radical scavenger	Improved functional outcome	(*170*, *171*)
	Tirilizad	Lipid peroxidation inhibitor	Lack of efficacy and toxic	(*172*, *173*)
	Ebselen	Free radical scavenger	No improvement of functional outcome at 3 months	(*174*)
Parkinson's Disease	Vitamin E	Free radical scavenger	Lack of efficacy	(*175–178*)
	Coenzyme Q (ubiquinone)	Mitochondrial associated electron carrier/free radical scavenger	Slows disease progression, improved symptoms	(*179*, *180*)
	glutathione	Non-enzyme scavenger	Mild symptomatic relief	(*181*, *182*)
	MitoQ	Mitochondrial associated electron carrier/free radical scavenger	Lack of efficacy	(*183*)

Continued on next page.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Table I. (Continued). Efficacy of some selected antioxidants in human studies

Neurodegenerative Disease	*Antioxidant*	*Mechanism of Action*	*Outcome*	*Reference*
Alzheimer's Disease	Vitamin E	Free radical scavenger	Slows disease progression or risk	(*184*, *185*)
			Does not reduce risk of developing AD	(*186–188*)
	Coenzyme Q (ubiquinone)	Mitochondrial associated electron carrier/free radical scavenger	Improved cognitive function; slowed disease progression	(*189*)(*190*)
			Lack of efficacy	(*191*)
Amyotrophic Lateral Sclerosis	Vitamin E	Free radical scavenger	No effect on rate of functional deterioration	(*192*, *193*)
			Lowers risk of developing ALS	(*194*)
	Edaravone	Free radical scavenger	Delays progression of functional motor disturbances	(*195*)
	KNS-760704	Free radical scavenger	Safe and well-tolerated	(*196*)
	Coenzyme Q (ubiquinone)	Mitochondrial associated electron carrier/free radical scavenger	Lack of efficacy	(*197*)

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

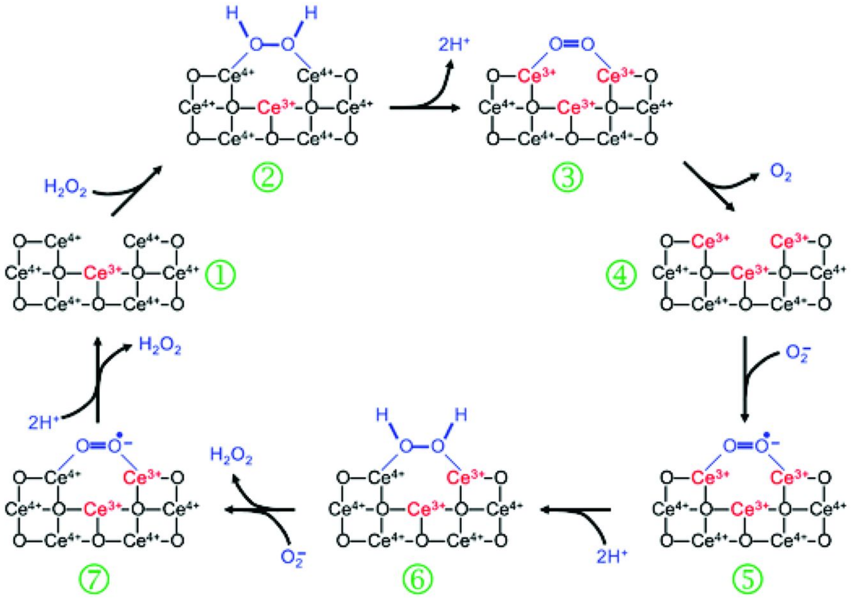


Figure 3. Model of the reaction mechanism for the oxidation of hydrogen peroxide by nanoceria and the regeneration via reduction by superoxide. An oxygen vacancy site on the nanoceria surface (1) presents a $2Ce^{4+}$ binding site for H_2O_2 (2), after the release of protons and two-electron transfer to the two cerium ions (3) oxygen is released from the now fully reduced oxygen vacancy site (4). Subsequently superoxide can bind to this site (5), and after the transfer of a single electron from one Ce^{3+}, and uptake of two protons from the solution, H_2O_2 is formed (6) and can be released. After repeating this reaction with a second superoxide molecule (7) the oxygen vacancy site returns to the initial $2Ce^{4+}$ state (1). It is also possible that the third Ce^{3+} indicated, which gives rise to the oxygen vacancy, could participate directly in the reaction mechanism. The square Ce–O matrix is shown here only to illustrate the model and does not correspond to the actual spatial arrangement of the atoms in the crystal structure. Figure and legend reproduced with permission from reference (201). Copyright 2011 Royal Society of Chemistry. (see color insert)

This ability of nanoceria to switch oxidation states and recycle their antioxidant activity is a unique advantage and has clear therapeutic implications. When combined with a catalytic activity which exceeds that of SOD (*166*) and an ability to cross the BBB (*202–205*), it becomes clear these particles have advantages for therapeutic use in oxidative stress disorders which overcome many of the shortcomings of previous therapies.

Although nanoceria have been widely used in industrial applications such as oxygen sensors (*206*) and automotive catalytic converters (*207*), they have recently begun to be used in biological systems (*25*, *202–204*). Importantly, focus has shifted from using these nanoparticles as drug delivery devices (e.g., (*208*)) to harnessing the inherent antioxidant capacity for therapeutic purposes. For example, nanoceria protected a hippocampal neuronal cell line from oxidative stress (*203*); decreased both NO and peroxynitrite formation in a murine model of ischemic cardiomyopathy (*209*); slowed the progression of retinal degeneration in a genetically susceptible mouse model (*210*); and inhibited the development and promoted the regression of retinal vascular lesions in a mouse model of age-related macular degeneration (*211*).

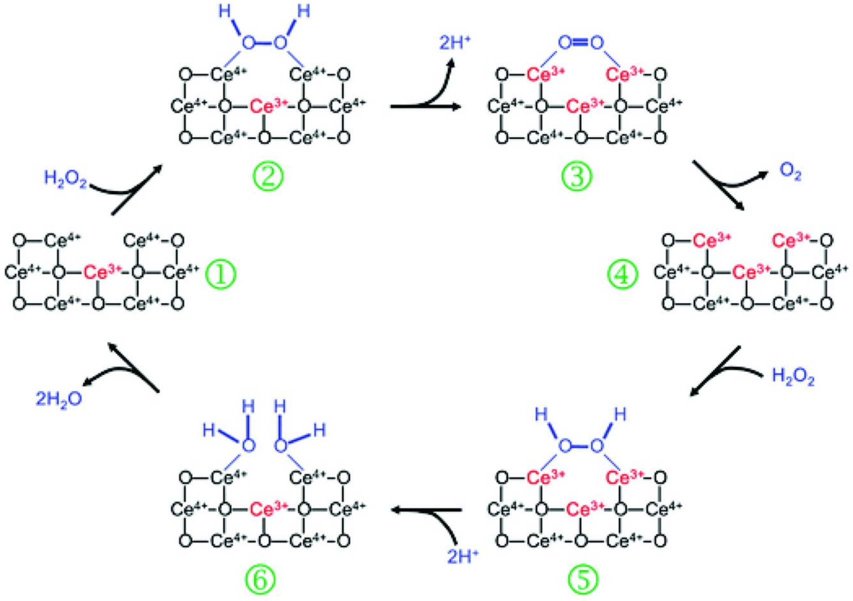


Figure 4. A model of the reaction mechanism for the complete dismutation of hydrogen peroxide. The oxidative half-reaction is identical to the sequence shown in Figure () (1–4). The reductive half involves binding of H_2O_2 to the $2Ce^{3+}$ site (5), uptake of two protons and homolysis of the O–O bond with transfer of electrons to the two Ce^{3+} (6), and release of the water molecules to regenerate the initial Ce^{4+} site (1). This reaction sequence would be analogous to the one found in catalases. Figure and legend reproduced with permission from reference (201). Copyright 2011 Royal Society of Chemisty. (see color insert)

Recently, our group looked into the mechanisms of nanoceria neuroprotection in a biological system (*212*). Using a hippocampal brain slice model of stroke, we showed that nanoceria were protective by moderately reducing $O_2^{\bullet-}$ and NO accumulation but significantly reducing tissue nitrosylation – a modification to protein residues induced by $ONOO^-$. These data suggest that reducing the effects of peroxynitrite is an important step in mitigating ischemia-induced cell death. $ONOO^-$ is both an oxidizing and nitrating agent that can induce inflammation and cell death via necrotic or apoptotic mechanisms (reviewed in (*50*)). In addition,

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

nitration of tyrosine residues can have profound impact on protein activity and can trigger an immune response (*49*). For example, Niu et al. demonstrated that treatment with nanoceria was protective in a mouse model of cardiomyopathy by several mechanisms including the inhibition of protein nitration and a decrease in inflammatory mediators (*209*). Nanoceria also decrease ROS production and inflammation in a mouse macrophage cell line (*213*). It is likely that the time course of generation and accumulation of ROS species may vary depending on the type of pathology. However, fact that nanoceria have the capacity to scavenge all biologically relevant ROS suggests that its therapeutic relevance may also extend beyond ischemic injury. Indeed, preliminary work from our group has shown that nanoceria are effective in decreasing motor deficits in the EAE model of relapsing multiple sclerosis (*205*).

Although nanoceria have profound free radical scavenging capabilities and have been shown to be non-toxic and protective (*25*, *203*, *212–214*) there are reports of toxicity in some studies (*215–219*). One proposed mechanism for this observed toxicity is increased oxidative stress (*217*, *220*, *221*) perhaps owing to the participation of nanoceria in Fenton reaction-like reactions in the presence of H_2O_2 (*222*).

These divergent results obtained with nanoceria, beneficial antioxidant in one case and toxic pro-oxidant in another, warrant further analysis. Arguably, cell-specific responses, methodological differences in the viability measurements made or the physical properties and synthetic route of the nanoparticles (some methods use organic solvents whereas other do not) used in the various studies can play a role. For example, exposure of human bronchial epithelial cells (BEAS-2B) to nanoceria reduced viability when measured using the 3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (*219*). MTT undergoes reductive cleavage by an incompletely understood cellular enzymatic system to yield a color precipitate in live cells. Because of its reliance on enzymatic processes, any intervention that alters one of the enzymes involved in dye cleavage may lead to misleading viability results (e.g., (*223*)). In light of this, it is interesting that a study using propidium iodide to measure viability demonstrated no toxic effects of nanoceria on a variety of cell lines, including BEAS-2B cells (*214*).

Moreover, surface properties such as size, charge, chemical coating, crystallinity and reactivity can also differ across particles (*224*, *225*) and these properties can influence among other things, the distribution of the particle to the target, adsorption of native proteins to the particle surface, or the propensity of the particle to agglormerate in a biological milieu. What is clear is that it is difficult to predict *a priori* whether a particular type of particle will be toxic based soley on its physical parameters. For example, a 6 nm particle exhibited ‘moderate’ toxicity to cultured cells (*218*) whereas as a different 5 nm particle showed no overt toxicity when givien to mice intravenously (*226*). In a toxicological analysis of several metal oxide nanoparticles, including CeO_2, Lanone et al. (2009) found no correlation between cytotoxicicty and spherical diameter or surface area. To confound matters, biological effects may differ from those observed in vitro. For example, Xia et al. (2008) observed that CeO_2 contributed to H_2O_2 generation in

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

vitro whereas it was protective against exogenous oxidative stress in transformed human bronchial epithelial cells as well as a phagocytic cell line.

In analyzing the whole animal effects of nanoceria and potentially translating these to human therapeutic use, the question of biodistribution of these particles is key since localization of the particles in an organism is an important determinant of toxicity and effectiveness. Yokel et al. (2009) examined the biodistribution of uncoated, 30 nm nanoceria in rats following i.v. infusion. Infusions were delivered to the rats via tail vein between 0.5–7.5 h, and the doses ranged from 50–750 mg/kg. The doses used in this study were far greater than those used in any previous studies given their interest in determining the biodistribution and toxicology of the cerium oxide nanoparticles. Importantly, few behavioral changes were noted aside from a slight tachypnea and an increase in chewing and licking demonstrated by some animals during infusion. No adverse effects were noted following the administration of the nanoceria, and none of the animals died prior to the end of the experiment. Up to 20 h following the infusions, the majority of ceria was distributed either in the liver or the spleen; less than 0.1% was located in the brain. The localization of ceria principally in the liver and spleen may have been due to the relative ease with which these particular particles agglomerated in vivo and the role of these two organ systems in detoxification of the blood. In contrast, a follow-up study by the same group (*216*) evaluated 5 nm, citrate-coated CeO_2 nanoparticles using a similar dosing regimen in rats and saw no distribution of the particles into the brain and increased toxicity – several rats died prior to the end of the experiment, presumably due to pulmonary toxicity. This was counterintuitive to what they expected since a smaller, coated nanoparticle was expected to be able to cross the blood brain barrier. The authors speculate that agglomeration of the small particles was responsible for the observed lung toxicity. A different study in mice looked at 5 nm uncoated CeO_2 particles and observed a similar biodistribution: spleen>liver>lung>kidneys (*226*). Interestingly, this nanoparticle displayed no blood brain barrier penetration and no overt toxicity. Finally, in preliminary studies in our lab, we have shown using ICP-MS that our custom-synthesized 2.5 nm citrate-stabilized particles penetrate the blood brain barrier both in healthy animals and animals with breakdown of the BBB due to disease. We performed this work using the SJL strain of mice which are frequently used to study the pathogensis of multiple sclerosis (EAE model). Using an identical dosing regimen, we were surprised to see that the deposition of ceria in the brain was very similar between healthy, controls compared to animals at the peak of their disease. Previous studies have repeatedly shown that there is significant breakdown of the BBB in the EAE model (*227*) consistent with the CNS effects of this disease model. Taken together, these data suggest that the movement of our nanoparticles into the brain is independent of BBB integrity suggesting that the flux of ceria particles into the brain vascular bed is likely due to both passive diffusion through tight junctions (~4 nm pore size) and active transport by endogenous carrier systems. This is significant given that few compounds currently under development are capable of crossing the BBB. Previous synthesis methods have not been able to produce uniform, monodispersed particles less than 5 nm and the nature of the citric acid stabilizer system we use appears to make the particles more biocompatible and better tolerated than particles developed by others. Importantly, administration of our particles weekly following the onset of disease greatly reduced the clinical severity and the motor deficits associated with the murine EAE model (*205*).

Table II. Comparison of CeO_2 nanoparitcles used in four recent studies

Parameter	*Yokel et al., 2009*	*Hardas et al., 2010*	*Hirst et al., 2011*	*DeCoteau et al., 2011*
Species	Rat	Rat	Mouse	Mouse
Size of CeO_2 nanoparticle	31 nm	5 nm	5 nm	2.5 nm
Coating of CeO_2 nanoparticle	none	Citrate alone	none	Citrate-based stabilizer
Cross BBB	Yes	No	No	Yes
Toxicity	None	Death of some rats due to pulmonary toxicity	None reported (although study not designed to check toxicity)	None

In an attempt to reconcile the varied biodistribution (Table II) and toxicity reports of nanoceria in whole animals, we speculate that factors associated with particle's ability to reach the intended site of therapeutic action rather than inherent in the catalytic activity of the particle itself are at work. That is, the toxic effects seen in some studies are most likely due to the off-target accumulation of the nanoceria (e.g., lung toxicity observed by Hardas et al. (*216*)) or particle-induced activation of the animal's immune system. In cases where these factors can be avoided, therapeutic effects and no toxicity are observed. For example, we observed neuroprotection and no toxicity in hippocampal brain slice model which used a commercially available, ~ 10 nm uncoated particle that was delivered directly to the target (a brain slice in culture) (*212*). Similarly, using custom-synthesized citrate-stabilized 2.5 nm particles we observed neuroprotection without toxicity with the particles able to make it to the target site (CNS) (*205*). Finally, Zhou et al. recently used 3-5 nm uncoated nanoceria administered directly at the target site (retina) and observed therapeutic effects with no reports of toxicity (*211*). In summary, these results suggest that toxicity or neuroprotection by nanoceria depends on factors that influence the particle's ability to get to the site of therapeutic action. The physical properties of the nanoparticle , including size, charge, coating and other surface characteristics, influence effectiveness or toxicity to the extent that they affect the biodistribution of the particles or interactions with off target sites (such as the lungs or immune

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

system). Thus, this will be an important consideration when attempting to translate the use of these particles from bench to bedside.

Conclusions

The most common neurodegenerative diseases all have similar pathophysiology involving oxidative stress and mitochondrial dysfunction. There is a lack of effective therapeutics to limit ROS damage in these pathologies. CeO_2 nanoparticles and other catalytic antioxidants present significant advantages over previously used therapies and represent the next generation of antioxidants for the treatment oxidative stress diseases. Specifically, CeO_2 nanoparticles exhibit high catalytic activity and a regenerative capacity to neutralize ROS. They can scavenge a broad range of ROS. The particles are small and can cross the blood brain barrier. Finally, a neuroprotective effect of nanoceria has been shown in many types of oxidative injury models. As nanoceria are modified to better ‘tune’ them for translation from bench to bedside, it is important that investigators be mindful of how changes in the physical parameters of the nanoparticle influence that particle’s ability to reach it’s intended therapeutic target in a whole animal.

References

1. Thannickal, V. J.; Fanburg, B. L. Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2000**, *279*, L1005–L1028.
2. Al Ghouleh, I.; Khoo, N. K. H.; Knaus, U. G.; Griendling, K. K.; Touyz, R. M.; Thannickal, V. J.; Barchowsky, A.; Nauseef, W. M.; Kelley, E. E.; Bauer, P. M.; Darley-Usmar, V.; Shiva, S.; Cifuentes-Pagano, E.; Freeman, B. A.; Gladwin, M. T.; Pagano, P. J. Oxidases and peroxidases in cardiovascular and lung disease: New concepts in reactive oxygen species signaling. *Free Radic. Biol. Med.* **2011**, *51*, 1271–1288.
3. Starkov, A. A. The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann. N. Y. Acad. Sci.* **2008**, *1147*, 37–52.
4. Coleman, J. W. Nitric oxide in immunity and inflammation. *Int. Immunopharmacol.* **2001**, *1*, 1397–1406.
5. Dale, D. C.; Boxer, L.; Liles, W. C. The phagocytes: Neutrophils and monocytes. *Blood* **2008**, *112*, 935–945.
6. Yin, J. X.; Yang, R. F.; Li, S.; Renshaw, A. O.; Li, Y. L.; Schultz, H. D.; Zimmerman, M. C. Mitochondria-produced superoxide mediates angiotensin II-induced inhibition of neuronal potassium current. *Am. J. Physiol. Cell. Physiol.* **2010**, *298*, C857–65.
7. Klann, E.; Roberson, E. D.; Knapp, L. T.; Sweatt, J. D. A role for superoxide in protein kinase C activation and induction of long-term potentiation. *J. Biol. Chem.* **1998**, *273*, 4516–4522.
8. Knapp, L. T.; Klann, E. Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C. *J. Neurosci.* **2002**, *22*, 674–683.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

9. Hopper, R. A.; Garthwaite, J. Tonic and phasic nitric oxide signals in hippocampal long-term potentiation. *J. Neurosci.* **2006**, *26*, 11513–11521.
10. Qiu, D. L.; Knopfel, T. An NMDA receptor/nitric oxide cascade in presynaptic parallel fiber-Purkinje neuron long-term potentiation. *J. Neurosci.* **2007**, *27*, 3408–3415.
11. Schrader, M.; Fahimi, H. D. Peroxisomes and oxidative stress. *Biochim. Biophys. Acta* **2006**, *1763*, 1755–1766.
12. Parker, M. W.; Blake, C. C. Iron- and manganese-containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Lett.* **1988**, *229*, 377–382.
13. Perry, J. J.; Shin, D. S.; Getzoff, E. D.; Tainer, J. A. The structural biochemistry of the superoxide dismutases. *Biochim. Biophys. Acta* **2010**, *1804*, 245–262.
14. Zamocky, M.; Koller, F. Understanding the structure and function of catalases: Clues from molecular evolution and in vitro mutagenesis. *Prog. Biophys. Mol. Biol.* **1999**, *72*, 19–66.
15. Goyal, M. M.; Basak, A. Human catalase: Looking for complete identity. *Protein Cell* **2010**, *1*, 888–897.
16. Arthur, J. R. The glutathione peroxidases. *Cell Mol. Life Sci.* **2000**, *57*, 1825–1835.
17. Brigelius-Flohe, R. Tissue-specific functions of individual glutathione peroxidases. *Free Radical Biol. Med.* **1999**, *27*, 951–965.
18. Bouayed, J.; Bohn, T. Exogenous antioxidants—Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid. Med. Cell. Longevity* **2010**, *3*, 228–237.
19. Diplock, A. T.; Charleux, J. L.; Crozier-Willi, G.; Kok, F. J.; Rice-Evans, C.; Roberfroid, M.; Stahl, W.; Vina-Ribes, J. Functional food science and defence against reactive oxidative species. *Br. J. Nutr.* **1998**, *80* (Supplement 1), S77–112.
20. Harman, D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **1956**, *11*, 298–300.
21. Sanz, A.; Stefanatos, R. K. The mitochondrial free radical theory of aging: A critical view. *Curr. Aging Sci.* **2008**, *1*, 10–21.
22. Lapointe, J.; Hekimi, S. When a theory of aging ages badly. *Cell Mol. Life Sci.* **2010**, *67*, 1–8.
23. Beckman, K. B.; Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **1998**, *78*, 547–581.
24. Carney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Landum, R. W.; Cheng, M. S.; Wu, J. F.; Floyd, R. A. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitrone. *Proc. Natl. Acad. Sci. U. S. A. U. S. A.* **1991**, *88*, 3633–3636.
25. Das, M.; Patil, S.; Bhargava, N.; Kang, J. F.; Riedel, L. M.; Seal, S.; Hickman, J. J. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials* **2007**, *28*, 1918–1925.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

26. Kim, J.; Takahashi, M.; Shimizu, T.; Shirasawa, T.; Kajita, M.; Kanayama, A.; Miyamoto, Y. Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of *Caenorhabditis elegans*. *Mech. Ageing Dev.* **2008**, *129*, 322–331.
27. Head, E. Oxidative damage and cognitive dysfunction: Antioxidant treatments to promote healthy brain aging. *Neurochem. Res.* **2009**, *34*, 670–678.
28. Calabrese, V.; Scapagnini, G.; Ravagna, A.; Colombrita, C.; Spadaro, F.; Butterfield, D. A.; Giuffrida Stella, A. M. Increased expression of heat shock proteins in rat brain during aging: relationship with mitochondrial function and glutathione redox state. *Mech. Ageing Dev.* **2004**, *125*, 325–335.
29. Rodrigues Siqueira, I.; Fochesatto, C.; da Silva Torres, I. L.; Dalmaz, C.; Alexandre Netto, C. Aging affects oxidative state in hippocampus, hypothalamus and adrenal glands of Wistar rats. *Life Sci.* **2005**, *78*, 271–278.
30. Sofic, E.; Sapcanin, A.; Tahirovic, I.; Gavrankapetanovic, I.; Jellinger, K.; Reynolds, G. P.; Tatschner, T.; Riederer, P. Antioxidant capacity in postmortem brain tissues of Parkinson's and Alzheimer's diseases. *J. Neural Transm., Suppl.* **2006** (71), 39–43.
31. Mariani, E.; Polidori, M. C.; Cherubini, A.; Mecocci, P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *J. Chromatogr., B* **2005**, *827*, 65–75.
32. Pratico, D. Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann. N. Y. Acad. Sci.* **2008**, *1147*, 70–78.
33. Chrissobolis, S.; Faraci, F. M. The role of oxidative stress and NADPH oxidase in cerebrovascular disease. *Trends Mol. Med.* **2008**, *14*, 495–502.
34. Danielson, S. R.; Andersen, J. K. Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radical Biol. Med.* **2008**, *44*, 1787–1794.
35. Miller, R. L.; James-Kracke, M.; Sun, G. Y.; Sun, A. Y. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **2009**, *34*, 55–65.
36. Niizuma, K.; Endo, H.; Chan, P. H. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J. Neurochem.* **2009**, *109* (Suppl 1), 133–138.
37. Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **2009**, *7*, 65–74.
38. Roger, V. L.; Go, A. S.; Lloyd-Jones, D. M.; Adams, R. J.; Berry, J. D.; Brown, T. M.; Carnethon, M. R.; Dai, S.; de Simone, G.; Ford, E. S.; Fox, C. S.; Fullerton, H. J.; Gillespie, C.; Greenlund, K. J.; Hailpern, S. M.; Heit, J. A.; Ho, P. M.; Howard, V. J.; Kissela, B. M.; Kittner, S. J.; Lackland, D. T.; Lichtman, J. H.; Lisabeth, L. D.; Makuc, D. M.; Marcus, G. M.; Marelli, A.; Matchar, D. B.; McDermott, M. M.; Meigs, J. B.; Moy, C. S.; Mozaffarian, D.; Mussolino, M. E.; Nichol, G.; Paynter, N. P.; Rosamond, W. D.; Sorlie, P. D.; Stafford, R. S.; Turan, T. N.; Turner, M. B.; Wong, N. D.; Wylie-Rosett, J. American Heart Association Statistics

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Committee and Stroke Statistics Subcommittee Heart disease and stroke statistics--2011 update: A report from the American Heart Association. *Circulation* **2011**, *123*, e18–e209.

39. Brouns, R.; De Deyn, P. P. The complexity of neurobiological processes in acute ischemic stroke. *Clin. Neurol. Neurosurg.* **2009**, *111*, 483–495.
40. Saver, J. L. Time is brain--Quantified. *Stroke* **2006**, *37*, 263–266.
41. Schild, L.; Reiser, G. Oxidative stress is involved in the permeabilization of the inner membrane of brain mitochondria exposed to hypoxia/reoxygenation and low micromolar Ca^{2+}. *FEBS J.* **2005**, *272*, 3593–3601.
42. Abramov, A. Y.; Scorziello, A.; Duchen, M. R. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J. Neurosci.* **2007**, *27*, 1129–1138.
43. Moro, M. A.; Almeida, A.; Bolanos, J. P.; Lizasoain, I. Mitochondrial respiratory chain and free radical generation in stroke. *Free Radical Biol. Med.* **2005**, *39*, 1291–1304.
44. Singh, I. N.; Sullivan, P. G.; Hall, E. D. Peroxynitrite-mediated oxidative damage to brain mitochondria: Protective effects of peroxynitrite scavengers. *J. Neurosci. Res.* **2007**, *85*, 2216–2223.
45. Suzuki, M.; Tabuchi, M.; Ikeda, M.; Tomita, T. Concurrent formation of peroxynitrite with the expression of inducible nitric oxide synthase in the brain during middle cerebral artery occlusion and reperfusion in rats. *Brain Res.* **2002**, *951*, 113–120.
46. Eliasson, M. J.; Huang, Z.; Ferrante, R. J.; Sasamata, M.; Molliver, M. E.; Snyder, S. H.; Moskowitz, M. A. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J. Neurosci.* **1999**, *19*, 5910–5918.
47. Endres, M.; Scott, G.; Namura, S.; Salzman, A. L.; Huang, P. L.; Moskowitz, M. A.; Szabo, C. Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(ADP-ribose) synthetase in a murine model of cerebral ischemia-reperfusion. *Neurosci. Lett.* **1998**, *248*, 41–44.
48. Gow, A. J.; Duran, D.; Malcolm, S.; Ischiropoulos, H. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett.* **1996**, *385*, 63–66.
49. Ischiropoulos, H. Protein tyrosine nitration--An update. *Arch. Biochem. Biophys.* **2009**, *484*, 117–121.
50. Szabo, C.; Ischiropoulos, H.; Radi, R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. *Nat. Rev. Drug Discovery* **2007**, *6*, 662–680.
51. Muralikrishna Adibhatla, R.; Hatcher, J. F. Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. *Free Radical Biol. Med.* **2006**, *40*, 376–387.
52. Phillis, J. W.; Horrocks, L. A.; Farooqui, A. A. Cyclooxygenases, lipoxygenases, and epoxygenases in CNS: Their role and involvement in neurological disorders. *Brain Res. Rev.* **2006**, *52*, 201–243.
53. Ono, T.; Tsuruta, R.; Fujita, M.; Aki, H. S.; Kutsuna, S.; Kawamura, Y.; Wakatsuki, J.; Aoki, T.; Kobayashi, C.; Kasaoka, S.; Maruyama, I.; Yuasa, M.; Maekawa, T. Xanthine oxidase is one of the major sources of

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

superoxide anion radicals in blood after reperfusion in rats with forebrain ischemia/reperfusion. *Brain Res.* **2009**, *1305*, 158–167.

54. Thiel, A.; Heiss, W. D. Imaging of microglia activation in stroke. *Stroke* **2011**, *42*, 507–512.
55. Wang, Q.; Tang, X. N.; Yenari, M. A. The inflammatory response in stroke. *J. Neuroimmunol.* **2007**, *184*, 53–68.
56. Colton, C. A.; Gilbert, D. L. Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.* **1987**, *223*, 284–288.
57. Yamato, M.; Egashira, T.; Utsumi, H. Application of in vivo ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. *Free Radical Biol. Med.* **2003**, *35*, 1619–1631.
58. Matsuda, S.; Umeda, M.; Uchida, H.; Kato, H.; Araki, T. Alterations of oxidative stress markers and apoptosis markers in the striatum after transient focal cerebral ischemia in rats. *J. Neural Transm.* **2009**, *116*, 395–404.
59. Oliver, C. N.; Starke-Reed, P. E.; Stadtman, E. R.; Liu, G. J.; Carney, J. M.; Floyd, R. A. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc. Natl. Acad. Sci. U. S. A. U. S. A.* **1990**, *87*, 5144–5147.
60. Floyd, R. A.; Carney, J. M. Free radical damage to protein and DNA: Mechanisms involved and relevant observations on brain undergoing oxidative stress. *Ann. Neurol.* **1992**, *32* (Supplement), S22–7.
61. Chong, Z. Z.; Li, F.; Maiese, K. Oxidative stress in the brain: Novel cellular targets that govern survival during neurodegenerative disease. *Prog. Neurobiol.* **2005**, *75*, 207–246.
62. Siegel, G. J.; Agranoff, B. W. In *Basic Neurochemistry: Molecular, Cellular, And Medical Aspects* Lippincott-Raven: Philadelphia, PA 1999.
63. Roth, J. M. Recombinant tissue plasminogen activator for the treatment of acute ischemic stroke. *Proceedings. (Baylor Univ. Med. Cent.)* **2011**, *24*, 257–259.
64. Fornai, F.; Lenzi, P.; Gesi, M.; Ferrucci, M.; Lazzeri, G.; Natale, G.; Ruggieri, S.; Paparelli, A. Recent knowledge on molecular components of Lewy bodies discloses future therapeutic strategies in Parkinson's disease. *Curr. Drug Targets: CNS Neurol. Disord.* **2003**, *2*, 149–152.
65. Spina, M. B.; Cohen, G. Dopamine turnover and glutathione oxidation: Implications for Parkinson disease. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 1398–1400.
66. Jackson-Lewis, V.; Smeyne, R. J. MPTP and SNpc DA neuronal vulnerability: Role of dopamine, superoxide and nitric oxide in neurotoxicity. *Neurotox. Res.* **2005**, *7*, 193–202.
67. Surmeier, D. J.; Guzman, J. N.; Sanchez-Padilla, J.; Goldberg, J. A. The origins of oxidant stress in Parkinson's disease and therapeutic strategies. *Antioxid. Redox Signal* **2011**, *14*, 1289–1301.
68. Dauer, W.; Przedborski, S. Parkinson's disease: Mechanisms and models. *Neuron* **2003**, *39*, 889–909.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

69. Schapira, A. H.; Cooper, J. M.; Dexter, D.; Clark, J. B.; Jenner, P.; Marsden, C. D. Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **1990**, *54*, 823–827.
70. Schapira, A. H.; Mann, V. M.; Cooper, J. M.; Dexter, D.; Daniel, S. E.; Jenner, P.; Clark, J. B.; Marsden, C. D. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. *J. Neurochem.* **1990**, *55*, 2142–2145.
71. Good, P. F.; Hsu, A.; Werner, P.; Perl, D. P.; Olanow, C. W. Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **1998**, *57*, 338–342.
72. Schober, A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res.* **2004**, *318*, 215–224.
73. Cleeter, M. W.; Cooper, J. M.; Schapira, A. H. Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: Evidence for free radical involvement. *J. Neurochem.* **1992**, *58*, 786–789.
74. Perumal, A. S.; Gopal, V. B.; Tordzro, W. K.; Cooper, T. B.; Cadet, J. L. Vitamin E attenuates the toxic effects of 6-hydroxydopamine on free radical scavenging systems in rat brain. *Brain Res. Bull.* **1992**, *29*, 699–701.
75. Davis, G. C.; Williams, A. C.; Markey, S. P.; Ebert, M. H.; Caine, E. D.; Reichert, C. M.; Kopin, I. J. Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. *Psychiatry Res.* **1979**, *1*, 249–254.
76. Ballard, P. A.; Tetrud, J. W.; Langston, J. W. Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): Seven cases. *Neurology* **1985**, *35*, 949–956.
77. Langston, J. W.; Ballard, P.; Tetrud, J. W.; Irwin, I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **1983**, *219*, 979–980.
78. LeWitt, P. A. Levodopa therapeutics for Parkinson's disease: New developments. *Parkinsonism Relat. Disord.* **2009**, *15*, S31–S34.
79. Braak, H.; Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* **1991**, *82*, 239–259.
80. Kukull, W. A.; Higdon, R.; Bowen, J. D.; McCormick, W. C.; Teri, L.; Schellenberg, G. D.; van Belle, G.; Jolley, L.; Larson, E. B. Dementia and Alzheimer disease incidence: A prospective cohort study. *Arch. Neurol.* **2002**, *59*, 1737–1746.
81. Evans, D. A.; Funkenstein, H. H.; Albert, M. S.; Scherr, P. A.; Cook, N. R.; Chown, M. J.; Hebert, L. E.; Hennekens, C. H.; Taylor, J. O. Prevalence of Alzheimer's disease in a community population of older persons. Higher than previously reported. *JAMA, J. Am. Med. Assoc.* **1989**, *262*, 2551–2556.
82. Mahley, R. W.; Weisgraber, K. H.; Huang, Y. Apolipoprotein E4: A causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 5644–5651.
83. Findeis, M. A. The role of amyloid beta peptide 42 in Alzheimer's disease. *Pharmacol. Ther.* **2007**, *116*, 266–286.
84. Hensley, K.; Carney, J. M.; Mattson, M. P.; Aksenova, M.; Harris, M.; Wu, J. F.; Floyd, R. A.; Butterfield, D. A. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 3270–3274.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

85. Panda, D.; Goode, B. L.; Feinstein, S. C.; Wilson, L. Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule-binding domains of tau. *Biochemistry* **1995**, *34*, 11117–11127.
86. Grundke-Iqbal, I.; Iqbal, K.; Tung, Y. C.; Quinlan, M.; Wisniewski, H. M.; Binder, L. I. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 4913–4917.
87. Chung, S. H. Aberrant phosphorylation in the pathogenesis of Alzheimer's disease. *BMB Rep.* **2009**, *42*, 467–474.
88. Marcus, D. L.; Thomas, C.; Rodriguez, C.; Simberkoff, K.; Tsai, J. S.; Strafaci, J. A.; Freedman, M. L. Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Exp. Neurol.* **1998**, *150*, 40–44.
89. Moreira, P. I.; Nunomura, A.; Nakamura, M.; Takeda, A.; Shenk, J. C.; Aliev, G.; Smith, M. A.; Perry, G. Nucleic acid oxidation in Alzheimer disease. *Free Radical Biol. Med.* **2008**, *44*, 1493–1505.
90. Smith, C. D.; Carney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyd, R. A.; Markesbery, W. R. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 10540–10543.
91. Smith, M. A.; Richey Harris, P. L.; Sayre, L. M.; Beckman, J. S.; Perry, G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **1997**, *17*, 2653–2657.
92. Sayre, L. M.; Zelasko, D. A.; Harris, P. L.; Perry, G.; Salomon, R. G.; Smith, M. A. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J. Neurochem.* **1997**, *68*, 2092–2097.
93. Yusuf, S.; Dagenais, G.; Pogue, J.; Bosch, J.; Sleight, P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart outcomes prevention evaluation study investigators. *N. Engl. J. Med.* **2000**, *342*, 154–160.
94. Butterfield, D. A.; Drake, J.; Pocernich, C.; Castegna, A. Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid β-peptide. *Trends Mol. Med.* **2001**, *7*, 548–554.
95. Sayre, L. M.; Zagorski, M. G.; Surewicz, W. K.; Krafft, G. A.; Perry, G. Mechanisms of neurotoxicity associated with amyloid beta deposition and the role of free radicals in the pathogenesis of Alzheimer's disease: A critical appraisal. *Chem. Res. Toxicol.* **1997**, *10*, 518–526.
96. Cras, P.; Kawai, M.; Siedlak, S.; Mulvihill, P.; Gambetti, P.; Lowery, D.; Gonzalez-DeWhitt, P.; Greenberg, B.; Perry, G. Neuronal and microglial involvement in beta-amyloid protein deposition in Alzheimer's disease. *Am. J. Pathol.* **1990**, *137*, 241–246.
97. Davis, R. E.; Miller, S.; Herrnstadt, C.; Ghosh, S. S.; Fahy, E.; Shinobu, L. A.; Galasko, D.; Thal, L. J.; Beal, M. F.; Howell, N.; Parker, W. D. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 4526–4531.

98. Hirai, K.; Aliev, G.; Nunomura, A.; Fujioka, H.; Russell, R. L.; Atwood, C. S.; Johnson, A. B.; Kress, Y.; Vinters, H. V.; Tabaton, M.; Shimohama, S.; Cash, A. D.; Siedlak, S. L.; Harris, P. L. R.; Jones, P. K.; Petersen, R. B.; Perry, G.; Smith, M. A. Mitochondrial abnormalities in Alzheimer's disease. *J. Neurosci.* **2001**, *21*, 3017–3023.
99. Manczak, M.; Park, B. S.; Jung, Y.; Reddy, P. H. Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: Implications for early mitochondrial dysfunction and oxidative damage. *NeuroMol. Med.* **2004**, *5*, 147–162.
100. Swerdlow, R. H.; Khan, S. M. The Alzheimer's disease mitochondrial cascade hypothesis: An update. *Exp. Neurol.* **2009**, *218*, 308–315.
101. Muller, W. E.; Eckert, A.; Kurz, C.; Eckert, G. P.; Leuner, K. Mitochondrial dysfunction: Common final pathway in brain aging and Alzheimer's disease--therapeutic aspects. *Mol. Neurobiol.* **2010**, *41*, 159–171.
102. Facecchia, K.; Fochesato, L. A.; Ray, S. D.; Stohs, S. J.; Pandey, S. Oxidative toxicity in neurodegenerative diseases: Role of mitochondrial dysfunction and therapeutic strategies. *J. Toxicol.* **2011**, *2011*, 683–728.
103. Manczak, M.; Anekonda, T. S.; Henson, E.; Park, B. S.; Quinn, J.; Reddy, P. H. Mitochondria are a direct site of Aβ accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Hum. Mol. Genet.* **2006**, *15*, 1437–1449.
104. Nunomura, A.; Perry, G.; Aliev, G.; Hirai, K.; Takeda, A.; Balraj, E. K.; Jones, P. K.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R. B.; Smith, M. A. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **2001**, *60*, 759–767.
105. Clark, T. A.; Lee, H. P.; Rolston, R. K.; Zhu, X.; Marlatt, M. W.; Castellani, R. J.; Nunomura, A.; Casadesus, G.; Smith, M. A.; Lee, H. G.; Perry, G. Oxidative stress and its implications for future treatments and management of Alzheimer disease. *Int. J. Biomed. Sci.* **2010**, *6*, 225–227.
106. Silva, D. F.; Esteves, A. R.; Oliveira, C. R.; Cardoso, S. M. Mitochondria: The common upstream driver of abeta and tau pathology in Alzheimer's disease. *Curr. Alzheimer Res.* **2011**.
107. Gauthier, S.; Scheltens, P. Can we do better in developing new drugs for Alzheimer's disease? *Alzheimer's Dementia* **2009**, *5*, 489–491.
108. Palmer, A. M. Neuroprotective therapeutics for Alzheimer's disease: Progress and prospects. *Trends Pharmacol. Sci.* **2011**, *32*, 141–147.
109. Poser, C. M. Onset symptoms of multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **1995**, *58*, 253–254.
110. McElroy, J. P.; Oksenberg, J. R. Multiple sclerosis genetics 2010. *Neurol. Clin.* **2011**, *29*, 219–231.
111. Sayetta, R. B. Theories of the etiology of multiple sclerosis: A critical review. *J. Clin. Lab. Immunol.* **1986**, *21*, 55–70.
112. van der Mei, I. A.; Simpson, S., Jr; Stankovich, J.; Taylor, B. V. Individual and joint action of environmental factors and risk of MS. *Neurol. Clin.* **2011**, *29*, 233–255.
113. Lublin, F. D. Adoptive transfer of murine relapsing experimental allergic encephalomyelitis. *Ann. Neurol.* **1985**, *17*, 188–190.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

114. Glabinski, A.; Tawsek, N. S.; Bartosz, G. Increased generation of superoxide radicals in the blood of MS patients. *Acta Neurol. Scand.* **1993**, *88*, 174–177.
115. Lin, R. F.; Lin, T. S.; Tilton, R. G.; Cross, A. H. Nitric oxide localized to spinal cords of mice with experimental allergic encephalomyelitis: An electron paramagnetic resonance study. *J. Exp. Med.* **1993**, *178*, 643–648.
116. Ruuls, S. R.; Bauer, J.; Sontrop, K.; Huitinga, I.; ’t Hart, B. A.; Dijkstra, C. D. Reactive oxygen species are involved in the pathogenesis of experimental allergic encephalomyelitis in Lewis rats. *J. Neuroimmunol.* **1995**, *56*, 207–217.
117. Weiss, N.; Miller, F.; Cazaubon, S.; Couraud, P. O. The blood-brain barrier in brain homeostasis and neurological diseases. *Biochim. Biophys. Acta* **2009**, *1788*, 842–857.
118. Bauer, J.; Berkenbosch, F.; Van Dam, A. M.; Dijkstra, C. D. Demonstration of interleukin-1 beta in Lewis rat brain during experimental allergic encephalomyelitis by immunocytochemistry at the light and ultrastructural level. *J. Neuroimmunol.* **1993**, *48*, 13–21.
119. Jacobs, C. A.; Baker, P. E.; Roux, E. R.; Picha, K. S.; Toivola, B.; Waugh, S.; Kennedy, M. K. Experimental autoimmune encephalomyelitis is exacerbated by IL-1 alpha and suppressed by soluble IL-1 receptor. *J. Immunol.* **1991**, *146*, 2983–2989.
120. Renno, T.; Krakowski, M.; Piccirillo, C.; Lin, J. Y.; Owens, T. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J. Immunol.* **1995**, *154*, 944–953.
121. Huppert, J.; Closhen, D.; Croxford, A.; White, R.; Kulig, P.; Pietrowski, E.; Bechmann, I.; Becher, B.; Luhmann, H. J.; Waisman, A.; Kuhlmann, C. R. Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB J.* **2010**, *24*, 1023–1034.
122. Kebir, H.; Kreymborg, K.; Ifergan, I.; Dodelet-Devillers, A.; Cayrol, R.; Bernard, M.; Giuliani, F.; Arbour, N.; Becher, B.; Prat, A. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **2007**, *13*, 1173–1175.
123. van der Goes, A.; Brouwer, J.; Hoekstra, K.; Roos, D.; van den Berg, T. K.; Dijkstra, C. D. Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J. Neuroimmunol.* **1998**, *92*, 67–75.
124. Bo, L.; Dawson, T. M.; Wesselingh, S.; Mork, S.; Choi, S.; Kong, P. A.; Hanley, D.; Trapp, B. D. Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann. Neurol.* **1994**, *36*, 778–786.
125. Liu, J. S.; Zhao, M. L.; Brosnan, C. F.; Lee, S. C. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am. J. Pathol.* **2001**, *158*, 2057–2066.
126. Smith, K. J.; Lassmann, H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* **2002**, *1*, 232–241.
127. McDonald, L. J.; Moss, J. Stimulation by nitric oxide of an NAD linkage to glyceraldehyde-3-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 6238–6241.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

128. Renganathan, M.; Cummins, T. R.; Waxman, S. G. Nitric oxide blocks fast, slow, and persistent Na+ channels in C-type DRG neurons by S-nitrosylation. *J. Neurophysiol.* **2002**, *87*, 761–775.

129. Muriel, P.; Castaneda, G.; Ortega, M.; Noel, F. Insights into the mechanism of erythrocyte Na+/K+-ATPase inhibition by nitric oxide and peroxynitrite anion. *J. Appl. Toxicol.* **2003**, *23*, 275–278.

130. Brown, G. C.; Borutaite, V. Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. *Biochim. Biophys. Acta* **2004**, *1658*, 44–49.

131. Fenyk-Melody, J. E.; Garrison, A. E.;; Brunnert, S. R.; Weidner, J. R.; Shen, F.; Shelton, B. A.; Mudgett, J. S. Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J. Immunol.* **1998**, *160*, 2940–2946.

132. Sahrbacher, U. C.; Lechner, F.; Eugster, H. P.; Frei, K.; Lassmann, H.; Fontana, A. Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **1998**, *28*, 1332–1338.

133. Willenborg, D. O.; Staykova, M.; Fordham, S.; O'Brien, N.; Linares, D. The contribution of nitric oxide and interferon gamma to the regulation of the neuro-inflammation in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **2007**, *191*, 16–25.

134. Yednock, T. A.; Cannon, C.; Fritz, L. C.; Sanchez-Madrid, F.; Steinman, L.; Karin, N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* **1992**, *356*, 63–66.

135. Han, M. H.; Hwang, S. I.; Roy, D. B.; Lundgren, D. H.; Price, J. V.; Ousman, S. S.; Fernald, G. H.; Gerlitz, B.; Robinson, W. H.; Baranzini, S. E.; Grinnell, B. W.; Raine, C. S.; Sobel, R. A.; Han, D. K.; Steinman, L. Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. *Nature* **2008**, *451*, 1076–1081.

136. Kizelsztein, P.; Ovadia, H.; Garbuzenko, O.; Sigal, A.; Barenholz, Y. Pegylated nanoliposomes remote-loaded with the antioxidant tempamine ameliorate experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **2009**, *213*, 20–25.

137. Mangas, A.; Covenas, R.; Bodet, D.; de Leon, M.; Duleu, S.; Geffard, M. Evaluation of the effects of a new drug candidate (GEMSP) in a chronic EAE model. *Int. J. Biol. Sci.* **2008**, *4*, 150–160.

138. Moriya, M.; Nakatsuji, Y.; Miyamoto, K.; Okuno, T.; Kinoshita, M.; Kumanogoh, A.; Kusunoki, S.; Sakoda, S. Edaravone, a free radical scavenger, ameliorates experimental autoimmune encephalomyelitis. *Neurosci. Lett.* **2008**, *440*, 323–326.

139. Barber, S. C.; Shaw, P. J. Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target. *Free Radical Biol. Med.* **2010**, *48*, 629–641.

140. Wong, P. C.; Rothstein, J. D.; Price, D. L. The genetic and molecular mechanisms of motor neuron disease. *Curr. Opin. Neurobiol.* **1998**, *8*, 791–799.

141. Costa, J.; Gomes, C.; de Carvalho, M. Diagnosis, pathogenesis and therapeutic targets in amyotrophic lateral sclerosis. *CNS Neurol. Disord.: Drug Targets* **2010**, *9*, 764–778.
142. Rosen, D. R.; Siddique, T.; Patterson, D.; Figlewicz, D. A.; Sapp, P.; Hentati, A.; Donaldson, D.; Goto, J.; O'Regan, J. P.; Deng, H. X. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **1993**, *362*, 59–62.
143. Robberecht, W. Genetics of amyotrophic lateral sclerosis. *J. Neurol.* **2000**, *247*, 2–6.
144. Cluskey, S.; Ramsden, D. B. Mechanisms of neurodegeneration in amyotrophic lateral sclerosis. *Mol. Pathol.* **2001**, *54*, 386–392.
145. Reaume, A. G.; Elliott, J. L.; Hoffman, E. K.; Kowall, N. W.; Ferrante, R. J.; Siwek, D. F.; Wilcox, H. M.; Flood, D. G.; Beal, M. F.; Brown, R. H., Jr; Scott, R. W.; Snider, W. D. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.* **1996**, *13*, 43–47.
146. Ferrante, R. J.; Browne, S. E.; Shinobu, L. A.; Bowling, A. C.; Baik, M. J.; MacGarvey, U.; Kowall, N. W.; Brown, R. H., Jr; Beal, M. F. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J. Neurochem.* **1997**, *69*, 2064–2074.
147. Shaw, P. J.; Ince, P. G.; Falkous, G.; Mantle, D. Oxidative damage to protein in sporadic motor neuron disease spinal cord. *Ann. Neurol.* **1995**, *38*, 691–695.
148. Shibata, N.; Nagai, R.; Uchida, K.; Horiuchi, S.; Yamada, S.; Hirano, A.; Kawaguchi, M.; Yamamoto, T.; Sasaki, S.; Kobayashi, M. Morphological evidence for lipid peroxidation and protein glycoxidation in spinal cords from sporadic amyotrophic lateral sclerosis patients. *Brain Res.* **2001**, *917*, 97–104.
149. Simpson, E. P.; Henry, Y. K.; Henkel, J. S.; Smith, R. G.; Appel, S. H. Increased lipid peroxidation in sera of ALS patients: a potential biomarker of disease burden. *Neurology* **2004**, *62*, 1758–1765.
150. Smith, R. G.; Henry, Y. K.; Mattson, M. P.; Appel, S. H. Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. *Ann. Neurol.* **1998**, *44*, 696–699.
151. Benatar, M. Lost in translation: Treatment trials in the SOD1 mouse and in human ALS. *Neurobiol. Dis.* **2007**, *26*, 1–13.
152. Habib, A. A.; Mitsumoto, H. Emerging drugs for amyotrophic lateral sclerosis. *Expert Opin. Emerg. Drugs* **2011**.
153. The Lancet. Neuroprotection: The end of an era? *The Lancet* **2006**, *368*, 1548–1548.
154. Stroke Therapy Academic Industry Roundtable II (STAIR-II). Recommendations for Clinical Trial Evaluation of Acute Stroke Therapies. *Stroke* **2001**, *32*, 1598–1606.
155. Lees, K. R.; Zivin, J. A.; Ashwood, T.; Davalos, A.; Davis, S. M.; Diener, H.; Grotta, J.; Lyden, P.; Shuaib, A.; Hårdemark, H.; Wasiewski, W. W. NXY-059 for acute ischemic stroke. *N. Engl. J. Med.* **2006**, *354*, 588–600.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

156. Diener, H.; Lees, K. R.; Lyden, P.; Grotta, J.; Davalos, A.; Davis, S. M.; Shuaib, A.; Ashwood, T.; Wasiewski, W.; Alderfer, V.; Hårdemark, H.; Rodichok, L. NXY-059 for the treatment of acute stroke. *Stroke* **2008**, *39*, 1751–1758.

157. De Keyser, J.; Sulter, G.; Luiten, P. G. Clinical trials with neuroprotective drugs in acute ischaemic stroke: Are we doing the right thing? *Trends Neurosci.* **1999**, *22*, 535–540.

158. Savitz, S. I. Cosmic Implications of NXY-059. *Stroke* **2009**, *40*, S115–S118.

159. Steinhubl, S. R. Why have antioxidants failed in clinical trials? *Am. J. Cardiol.* **2008**, *101*, S14–S19.

160. Kamat, C. D.; Gadal, S.; Mhatre, M.; Williamson, K. S.; Pye, Q. N.; Hensley, K. Antioxidants in central nervous system diseases: Preclinical promise and translational challenges. *J. Alzheimers Dis.* **2008**, *15*, 473–493.

161. Lapchak, P. A. A critical assessment of edaravone acute ischemic stroke efficacy trials: Is edaravone an effective neuroprotective therapy? *Expert Opin. Pharmacother.* **2010**, *11*, 1753–1763.

162. Watanabe, T.; Tahara, M.; Todo, S. The novel antioxidant edaravone: From bench to bedside. *Cardiovasc. Ther.* **2008**, *26*, 101–114.

163. Banno, M.; Mizuno, T.; Kato, H.; Zhang, G.; Kawanokuchi, J.; Wang, J.; Kuno, R.; Jin, S.; Takeuchi, H.; Suzumura, A. The radical scavenger edaravone prevents oxidative neurotoxicity induced by peroxynitrite and activated microglia. *Neuropharmacology* **2005**, *48*, 283–290.

164. Yoshida, H.; Yanai, H.; Namiki, Y.; Fukatsu-Sasaki, K.; Furutani, N.; Tada, N. Neuroprotective effects of edaravone: A novel free radical scavenger in cerebrovascular injury. *CNS Drug Rev.* **2006**, *12*, 9–20.

165. Crow, J. P. Catalytic antioxidants to treat amyotropic lateral sclerosis. *Expert Opin. Invest. Drugs* **2006**, *15*, 1383–1393.

166. Korsvik, C.; Patil, S.; Seal, S.; Self, W. T. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem. Commun.* **2007**, *10*, 1056–1058.

167. Pirmohamed, T.; Dowding, J. M.; Singh, S.; Wasserman, B.; Heckert, E.; Karakoti, A. S.; King, J. E.; Seal, S.; Self, W. T. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem. Commun.* **2010**, *46*, 2736–2738.

168. Liang, L.; Huang, J.; Fulton, R.; Day, B. J.; Patel, M. An orally active catalytic metalloporphyrin protects against 1-methyl-4-Phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in vivo. *J. Neurosci.* **2007**, *27*, 4326–4333.

169. Shuaib, A.; Lees, K. R.; Lyden, P.; Grotta, J.; Davalos, A.; Davis, S. M.; Diener, H. C.; Ashwood, T.; Wasiewski, W. W.; Emeribe, U. SAINT II Trial Investigators NXY-059 for the treatment of acute ischemic stroke. *N. Engl. J. Med.* **2007**, *357*, 562–571.

170. Edaravone Acute Infarction Study Group. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. *Cerebrovasc. Dis.* **2003**, *15*, 222–229.

171. Sharma, P.; Sinha, M.; Shukla, R.; Garg, R. K.; Verma, R.; Singh, M. K. A randomized controlled clinical trial to compare the safety and efficacy of

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

edaravone in acute ischemic stroke. *Ann. Indian Acad. Neurol.* **2011**, *14*, 103–106.

172. Tirilazad International Steering Committee. Tirilazad mesylate in acute ischemic stroke: A systematic review. *Stroke* **2000**, *31*, 2257–2265.
173. RANTTAS Investigators. A randomized trial of tirilazad mesylate in patients with acute stroke (RANTTAS). *Stroke* **1996**, *27*, 1453–1458.
174. Yamaguchi, T.; Sano, K.; Takakura, K.; Saito, I.; Shinohara, Y.; Asano, T.; Yasuhara, H. Ebselen in acute ischemic stroke: A placebo-controlled, double-blind clinical trial. *Stroke* **1998**, *29*, 12–17.
175. Kieburtz, K.; McDermott, M.; Como, P.; Growdon, J.; Brady, J.; Carter, J.; Huber, S.; Kanigan, B.; Landow, E.; Rudolph, A. The effect of deprenyl and tocopherol on cognitive performance in early untreated Parkinson's disease. Parkinson Study Group. *Neurology* **1994**, *44*, 1756–1759.
176. Shoulson, I. DATATOP: A decade of neuroprotective inquiry. Parkinson Study Group. Deprenyl and tocopherol antioxidative therapy of Parkinsonism. *Ann. Neurol.* **1998**, *44*, S160–6.
177. The Parkinson Study Group. Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. *N. Engl. J. Med.* **1993**, *328*, 176–183.
178. Zhang, S. M.; Hernan, M. A.; Chen, H.; Spiegelman, D.; Willett, W. C.; Ascherio, A. Intakes of vitamins E and C, carotenoids, vitamin supplements, and PD risk. *Neurology* **2002**, *59*, 1161–1169.
179. Shults, C. W.; Oakes, D.; Kieburtz, K.; Beal, M. F.; Haas, R.; Plumb, S.; Juncos, J. L.; Nutt, J.; Shoulson, I.; Carter, J.; Kompoliti, K.; Perlmutter, J. S.; Reich, S.; Stern, M.; Watts, R. L.; Kurlan, R.; Molho, E.; Harrison, M.; Lew, M. Parkinson Study Group. Effects of coenzyme Q10 in early Parkinson disease: Evidence of slowing of the functional decline. *Arch. Neurol.* **2002**, *59*, 1541–1550.
180. Weber, C. A.; Ernst, M. E. Antioxidants, supplements, and Parkinson's disease. *Ann. Pharmacother.* **2006**, *40*, 935–938.
181. Sechi, G.; Deledda, M. G.; Bua, G.; Satta, W. M.; Deiana, G. A.; Pes, G. M.; Rosati, G. Reduced intravenous glutathione in the treatment of early Parkinson's disease. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **1996**, *20*, 1159–1170.
182. Hauser, R. A.; Lyons, K. E.; McClain, T.; Carter, S.; Perlmutter, D. Randomized, double-blind, pilot evaluation of intravenous glutathione in Parkinson's disease. *Mov. Disord.* **2009**, *24*, 979–983.
183. Snow, B. J.; Rolfe, F. L.; Lockhart, M. M.; Frampton, C. M.; O'Sullivan, J. D.; Fung, V.; Smith, R. A. J.; Murphy, M. P.; Taylor, K. M. A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease. *Mov. Disord.* **2010**, *25*, 1670–1674.
184. Sano, M.; Ernesto, C.; Thomas, R. G.; Klauber, M. R.; Schafer, K.; Grundman, M.; Woodbury, P.; Growdon, J.; Cotman, C. W.; Pfeiffer, E.; Schneider, L. S.; Thal, L. J. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N. Engl. J. Med.* **1997**, *336*, 1216–1222.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

185. Morris, M. C.; Beckett, L. A.; Scherr, P. A.; Hebert, L. E.; Bennett, D. A.; Field, T. S.; Evans, D. A. Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* **1998**, *12*, 121–126.

186. Luchsinger, J. A.; Tang, M. X.; Shea, S.; Mayeux, R. Antioxidant vitamin intake and risk of Alzheimer disease. *Arch. Neurol.* **2003**, *60*, 203–208.

187. Zandi, P. P.; Anthony, J. C.; Khachaturian, A. S.; Stone, S. V.; Gustafson, D.; Tschanz, J. T.; Norton, M. C.; Welsh-Bohmer, K. A.; Breitner, J. C. Cache County Study Group. Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: The Cache County study. *Arch. Neurol.* **2004**, *61*, 82–88.

188. Yaffe, K.; Clemons, T. E.; McBee, W. L.; Lindblad, A. S. Age-Related Eye Disease Study Research Group. Impact of antioxidants, zinc, and copper on cognition in the elderly: A randomized, controlled trial. *Neurology* **2004**, *63*, 1705–1707.

189. Gutzmann, H.; Kuhl, K. P.; Hadler, D.; Rapp, M. A. Safety and efficacy of idebenone versus tacrine in patients with Alzheimer's disease: Results of a randomized, double-blind, parallel-group multicenter study. *Pharmacopsychiatry* **2002**, *35*, 12–18.

190. Bergamasco, B.; Scarzella, L.; La Commare, P. Idebenone, a new drug in the treatment of cognitive impairment in patients with dementia of the Alzheimer type. *Funct. Neurol.* **1994**, *9*, 161–168.

191. Thal, L. J.; Grundman, M.; Berg, J.; Ernstrom, K.; Margolin, R.; Pfeiffer, E.; Weiner, M. F.; Zamrini, E.; Thomas, R. G. Idebenone treatment fails to slow cognitive decline in Alzheimer's disease. *Neurology* **2003**, *61*, 1498–1502.

192. Desnuelle, C.; Dib, M.; Garrel, C.; Favier, A. A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol Study Group. *Amyotroph Lateral Scler. Other Mot. Neuron Disord.* **2001**, *2*, 9–18.

193. Graf, M.; Ecker, D.; Horowski, R.; Kramer, B.; Riederer, P.; Gerlach, M.; Hager, C.; Ludolph, A. C.; Becker, G.; Osterhage, J.; Jost, W. H.; Schrank, B.; Stein, C.; Kostopulos, P.; Lubik, S.; Wekwerth, K.; Dengler, R.; Troeger, M.; Wuerz, A.; Hoge, A.; Schrader, C.; Schimke, N.; Krampfl, K.; Petri, S.; Zierz, S.; Eger, K.; Neudecker, S.; Traufeller, K.; Sievert, M.; Neundorfer, B.; Hecht, M. German Vitamin E/ALS Study Group. High dose vitamin E therapy in amyotrophic lateral sclerosis as add-on therapy to riluzole: results of a placebo-controlled double-blind study. *J. Neural Transm.* **2005**, *112*, 649–660.

194. Ascherio, A.; Weisskopf, M. G.; O'reilly, E. J.; Jacobs, E. J.; McCullough, M. L.; Calle, E. E.; Cudkowicz, M.; Thun, M. J. Vitamin E intake and risk of amyotrophic lateral sclerosis. *Ann. Neurol.* **2005**, *57*, 104–110.

195. Yoshino, H.; Kimura, A. Investigation of the therapeutic effects of edaravone, a free radical scavenger, on amyotrophic lateral sclerosis (Phase II study). *Amyotroph. Lateral. Scler.* **2006**, *7*, 241–245.

196. Bozik, M. E.; Mather, J. L.; Kramer, W. G.; Gribkoff, V. K.; Ingersoll, E. W. Safety, tolerability, and pharmacokinetics of KNS-760704 (dexpramipexole) in healthy adult subjects. *J. Clin. Pharmacol.* **2011**, *51*, 1177–1185.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

197. Kaufmann, P.; Thompson, J. L.; Levy, G.; Buchsbaum, R.; Shefner, J.; Krivickas, L. S.; Katz, J.; Rollins, Y.; Barohn, R. J.; Jackson, C. E.; Tiryaki, E.; Lomen-Hoerth, C.; Armon, C.; Tandan, R.; Rudnicki, S. A.; Rezania, K.; Sufit, R.; Pestronk, A.; Novella, S. P.; Heiman-Patterson, T.; Kasarskis, E. J.; Pioro, E. P.; Montes, J.; Arbing, R.; Vecchio, D.; Barsdorf, A.; Mitsumoto, H.; Levin, B. QALS Study Group. Phase II trial of CoQ10 for ALS finds insufficient evidence to justify phase III. *Ann. Neurol.* **2009**, *66*, 235–244.

198. Heckert, E. G.; Karakoti, A. S.; Seal, S.; Self, W. T. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials* **2008**, *29*, 2705–2709.

199. Robinson, R. D.; Spanier, J. E.; Zhang, F.; Chan, S.; Herman, I. P. Visible thermal emission from sub-band-gap laser excited cerium dioxide particles. *J. Appl. Phys.* **2002**, *92*, 1936–1941.

200. Esch, F.; Fabris, S.; Zhou, L.; Montini, T.; Africh, C.; Fornasiero, P.; Comelli, G.; Rosei, R. Electron localization determines defect formation on ceria substrates. *Science* **2005**, *309*, 752–755.

201. Celardo, I.; Pedersen, J. Z.; Traversa, E.; Ghibelli, L. Pharmacological potential of cerium oxide nanoparticles. *Nanoscale* **2011**, *3*, 1411–1420.

202. Chen, J.; Patil, S.; Seal, S.; McGinnis, J. F. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nat. Nanotechnol.* **2006**, *1*, 142–150.

203. Schubert, D.; Dargusch, R.; Raitano, J.; Chan, S. W. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem. Biophys. Res. Commun.* **2006**, *342*, 86–91.

204. Silva, G. A. Nanomedicine: Seeing the benefits of ceria. *Nat. Nanotechnol.* **2006**, *1*, 92–94.

205. DeCoteau, W. E.; Estevez, A. Y.; Leo-Nyquist, S.; Heckman, K.; Reed, K.; Erlichman, J. S. Ceria Nanoparticles Reduce Disease Severity in a Mouse Model of Multiple Sclerosis. TechConnect World Conference and Expo, Boston, MA, June 13−16, 2011.

206. Izu, N.; Shin, W.; Matsubara, I.; Murayama, N. Development of resistive oxygen sensors based on cerium oxide thick film. *J. Electroceram.* **2004**, *13*, 703–706.

207. Masui, T.; Ozaki, T.; Machida, K.; Adachi, G. Preparation of ceria–zirconia sub-catalysts for automotive exhaust cleaning. *J. Alloys Compd.* **2000**, *303-304*, 49–55.

208. Patil, S.; Reshetnikov, S.; Haldar, M. K.; Seal, S.; Mallik, S.; Aarts, M. Surface-derivatized nanoceria with human carbonic anhydrase ii inhibitors and fluorophores: A potential drug delivery device. *J. Phys. Chem.* **2007**, *111*, 8437.

209. Niu, J.; Azfer, A.; Rogers, L. M.; Wang, X.; Kolattukudy, P. E. Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy. *Cardiovasc. Res.* **2007**, *73*, 549–559.

210. Kong, L.; Cai, X.; Zhou, X.; Wong, L. L.; Karakoti, A. S.; Seal, S.; McGinnis, J. F. Nanoceria extend photoreceptor cell lifespan in tubby mice

by modulation of apoptosis/survival signaling pathways. *Neurobiol. Dis.* **2011**, *42*, 514–523.

211. Zhou, X.; Wong, L. L.; Karakoti, A. S.; Seal, S.; McGinnis, J. F. Nanoceria inhibit the development and promote the regression of pathologic retinal neovascularization in the *Vldlr* knockout mouse. *PLoS ONE* **2011**, *6*, e16733.
212. Estevez, A. Y.; Pritchard, S.; Harper, K.; Aston, J. W.; Lynch, A.; Lucky, J. J.; Ludington, J. S.; Chatani, P.; Mosenthal, W. P.; Leiter, J. C.; Andreescu, S.; Erlichman, J. S. Neuroprotective mechanisms of cerium oxide nanoparticles in a mouse hippocampal brain slice model of ischemia. *Free Radical Biol. Med.* **2011**, *51*, 1155–1163.
213. Hirst, S. M.; Karakoti, A. S.; Tyler, R. D.; Sriranganathan, N.; Seal, S.; Reilly, C. M. Anti-inflammatory properties of cerium oxide nanoparticles. *Small* **2009**, *5*, 2848–2856.
214. Xia, T.; Kovochich, M.; Liong, M.; Madler, L.; Gilbert, B.; Shi, H.; Yeh, J. I.; Zink, J. I.; Nel, A. E. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* **2008**, *2*, 2121–2134.
215. Lanone, S.; Rogerieux, F.; Geys, J.; Dupont, A.; Maillot-Marechal, E.; Boczkowski, J.; Lacroix, G.; Hoet, P. Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. *Part. Fibre Toxicol.* **2009**, *6*, 14.
216. Hardas, S. S.; Butterfield, D. A.; Sultana, R.; Tseng, M. T.; Dan, M.; Florence, R. L.; Unrine, J. M.; Graham, U. M.; Wu, P.; Grulke, E. A.; Yokel, R. A. Brain distribution and toxicological evaluation of a systemically delivered engineered nanoscale ceria. *Toxicol. Sci.* **2010**, *116*, 562–576.
217. Zhang, H.; He, X.; Zhang, Z.; Zhang, P.; Li, Y.; Ma, Y.; Kuang, Y.; Zhao, Y.; Chai, Z. Nano-CeO_2 exhibits adverse effects at environmental relevant concentrations. *Environ. Sci. Technol.* **2011**, *45*, 3725–3730.
218. Brunner, T. J.; Wick, P.; Manser, P.; Spohn, P.; Grass, R. N.; Limbach, L. K.; Bruinink, A.; Stark, W. J. In vitro cytotoxicity of oxide nanoparticles: Comparison to asbestos, silica, and the effect of particle solubility. *Environ. Sci. Technol.* **2006**, *40*, 4374–4381.
219. Eom, H. J.; Choi, J. Oxidative stress of CeO2 nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B. *Toxicol. Lett.* **2009**, *187*, 77–83.
220. Auffan, M.; Rose, J.; Orsiere, T.; De Meo, M.; Thill, A.; Zeyons, O.; Proux, O.; Masion, A.; Chaurand, P.; Spalla, O.; Botta, A.; Wiesner, M. R.; Bottero, J. CeO2 nanoparticles induce DNA damage towards human dermal fibroblasts in vitro. *Nanotoxicology* **2009**, *3*, 161–171.
221. Park, E. J.; Choi, J.; Park, Y. K.; Park, K. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* **2008**, *245*, 90–100.
222. Heckert, E. G.; Seal, S.; Self, W. T. Fenton-like reaction catalyzed by the rare earth inner transition metal cerium. *Environ. Sci. Technol.* **2008**, *42*, 5014–5019.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

223. Pozzolini, M.; Scarfi, S.; Benatti, U.; Giovine, M. Interference in MTT cell viability assay in activated macrophage cell line. *Anal. Biochem.* **2003**, *313*, 338–341.

224. Yokel, R. A.; Florence, R. L.; Unrine, J. M.; Tseng, M. T.; Graham, U. M.; Wu, P.; Grulke, E. A.; Sultana, R.; Hardas, S. S.; Butterfield, D. A. Biodistribution and oxidative stress effects of a systemically-introduced commercial ceria engineered nanomaterial. *Nanotoxicology* **2009**, *3*, 234–248.

225. Safi, M.; Sarrouj, H.; Sandre, O.; Mignet, N.; Berret, J. F. Interactions between sub-10-nm iron and cerium oxide nanoparticles and 3T3 fibroblasts: The role of the coating and aggregation state. *Nanotechnology* **2010**, *21*, 145103.

226. Hirst, S. M.; Karakoti, A.; Singh, S.; Self, W.; Tyler, R.; Seal, S.; Reilly, C. M. Bio-distribution and in vivo antioxidant effects of cerium oxide nanoparticles in mice. *Environ. Toxicol.* **2011**, DOI: 10.1002/tox.20704.

227. Pan, W.; Banks, W. A.; Kennedy, M. K.; Gutierrez, E. G.; Kastin, A. J. Differential permeability of the BBB in acute EAE: Enhanced transport of TNT-alpha. *Am. J. Physiol. Endocrinol. Metab.* **1996**, *271*, E636–E642.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch009

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Chapter 10

Detection of Superoxide and Hydrogen Peroxide from Living Cells Using Electrochemical Sensors

Szilveszter Gáspár[*]

**International Centre of Biodynamics, 1B Intrarea Portocalelor,
060101 Bucharest, Romania
[*]E-mail: sgaspar@biodyn.ro**

Superoxide and hydrogen peroxide are present in very low concentrations and are characterized by short half-life in the extra- and intracellular space of living cells because of a complex antioxidant system, which spans over both spaces and very efficiently consumes these species. Monitoring superoxide and hydrogen peroxide produced by living cells in real-time is thus rather challenging. Appropriately constructed electrochemical sensors and biosensors can continuously monitor superoxide or hydrogen peroxide produced by cells. As such, they can advantageously complete analytical methods which are offering endpoints and/or indirect evidence of the overproduction of these species by cells. The present chapter reviews electrochemical sensors and biosensors used to detect superoxide and hydrogen peroxide from cells. Although plenty of electrochemical sensors for superoxide and hydrogen peroxide were reported in the scientific literature, relatively few publications describe their use in cell biology. Moreover, most of these publications do not describe in depth studies but initiatives which stop at the proof of principle level instead.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

1. Introduction

Reactive oxygen species (such as superoxide and hydrogen peroxide, the two species the present chapter is focused on) play an important role in the physiology of living cells. Phagocytes (both macrophages and neutrophils) release reactive oxygen species in their fight with intruders (*1*). Hydrogen peroxide was found to activate a variety of vascular signaling pathways, leading to vascular contraction and growth (*2*). Hydrogen peroxide plays an important role also in "wound-to-leukocyte signaling" (*3*). Its concentration in the tissue increases in minutes after wounding and this leads to the recruitment of leukocytes to the site of injury.

Reactive oxygen species are also part of the biochemistry of several diseases and of the complex reaction of living cells to chemical and physical stress. Zinc, for example, was found to activate the NADPH oxidase of neutrophils via protein kinase C, leading to the formation of superoxide (*4*). The overproduction of reactive oxygen species (a phenomenon called oxidative stress) plays an important role in the pathogenesis of several neurodegenerative diseases (*5*) and cardiovascular diseases (*6*) (to mention just two among many other important classes of diseases).

The half-life of superoxide and how far superoxide diffuses from its source in different biological systems seem to be still disputed. The half-life was reported to be below 50 milliseconds, in the presence of various blood constituents (*7*). Such a half-life will allow superoxide to diffuse about 40 μm from the source of its production. Other studies build on a lifetime of 1 μs and the fact that superoxide cannot diffuse deeper than 45 nm in a collagen film (*8*). The half-life of superoxide is clearly short and dependent on the chemical composition of the microenvironment superoxide is generated into.

Data seem to converge more pronouncedly regarding the half-life of hydrogen peroxide in biological systems. When incubated with lung perfusates, the half-life of hydrogen peroxide was found to be 0.31 minutes (*9*). A slightly longer half-life (4 minutes) was reported in less complex environments such as the incubation buffer of some astroglial cells (*10*). The half-life of hydrogen peroxide is clearly longer than that of superoxide. However, is still short compared to the long incubation times (i.e. hours) which are commonly used when the effect of a compound on cells is investigated in biology.

In addition to their relatively short half-life, superoxide and hydrogen peroxide are produced in really small amounts by living cells. For example, the concentration of superoxide and hydrogen peroxide in phorbol myristate acetate-stimulated macrophage cell extracts was found to be 0.78 and 1.14 μM, respectively (*11*). Phorbol myristate acetate is a protein kinase C agonist that activates membrane-bound NADPH oxidase resulting in superoxide release (*12*). Macrophages are cells specialized to deliberately produce important quantities of reactive oxygen species, and thus the generation of even lower amounts of superoxide and hydrogen peroxide can be expected from other cell types.

Detection of superoxide and/or hydrogen peroxide from living cells is further complicated by the fact that, in aqueous solutions, superoxide undergoes disproportionation that gives hydrogen peroxide. The disproportionation reaction

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

is second order with respect to superoxide and is very fast (k = 2 x 10^5 M^{-1} s^{-1} at 25 °C and pH of 7.4 (*13*)). Separation of the biological effect of superoxide and hydrogen peroxide, although of great scientific interest, is thus not an easy task.

Analytical tools to detect superoxide and hydrogen peroxide from living cells must clearly have great selectivity, short response times and must ideally be small enough to be placed in the close proximity of the investigated cells. The reason for the last requirement is a very simple one. Small amounts of superoxide or hydrogen peroxide released by cells can still lead to high concentrations (and satisfactory signal-to-noise ratios) as long as the volume these molecules are released into is kept small. Only few such analytical tools emerged and many cell biologists are still investigating oxidative stress based on indirect evidence of the overproduction of reactive oxygen species (e.g. by detecting stable oxidation products).

The present chapter reviews electrochemical sensors already used to detect superoxide or hydrogen peroxide generated by living cells. It is intended to update some other excellent reviews on the subject (*13*, *14*) with the progresses registered during the last few years. Although they pave the way to the application of such sensors at cellular level, papers detailing sensor development and characterization (but not their use at cellular level) are not mentioned for the sake of conciseness. There are other reviews on that matter (*15*, *16*).

2. Electrochemical Sensors and Biosensors for Detection of Superoxide

There are two types of electrochemical sensors used to detect superoxide produced by living cells (see Table I for few examples). The standard redox potential of the oxygen/superoxide redox couple (- 330 mV vs NHE according to some sources (*13*), and - 137 mV vs. NHE according to other sources (*17*)) allows detection of superoxide by direct oxidation on unmodified electrodes. Therefore, the first type of electrochemical sensor for the detection of superoxide is actually a simple, unmodified, metal electrode. The second type of electrochemical sensor for the detection of superoxide uses redox proteins which are able to selectively convert superoxide and which are immobilized on the electrode surface. In such sensors, after the reaction with superoxide, the immobilized redox protein is oxidized or reduced back to its initial redox state by direct electron transfer to/from the electrode, that is, the immobilized redox protein acts as an electron transfer mediator. The selectivity of the redox protein – substrate reaction is thus advantageously added to the sensitivity of the electrochemical detection.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Table I. Design details of some electrochemical sensors and biosensors already used for the detection of superoxide from cells

Electrode	*Biorecognition element*	*Immobilization method*	*Working potential / mV*	*Ref.*
6 mm diameter pyrolytic graphite disk	-	-	+ 50	(*18*)
10 µm diameter gold disk	-	-	+ 50	(*19*)
1 - 2 mm diameter gold disk	Cytochrome c	Covalent immobilization to a SAM[1] of 3,3′-dithiobis(sulfosuccinimidylpropionate)	+ 100	(*24*, *29*)
7 µm diameter carbon fiber	-	-	+ 100	(*20*)
0.9 mm diameter gold disk	Pseudomonas aeruginosa azurin	Covalent immobilization to a SAM of 3,3′-dithiobis(sulfosuccinimidylpropionate)	+ 250	(*34*)
3 mm diameter gold disk	Superoxide dismutase	Covalent immobilization to a SAM of cysteine	+ 300	(*32*)
0.8 mm diameter, 5 mm high gold cylinder	Cytochrome c	Covalent immobilization to a SAM of 3,3′-dithiodipropionic acid	+ 150	(*30*)
0.5 mm diameter platinum disk	Superoxide dismutase	Cross-linking with glutaraldehyde onto a carbon nanotube poly(pyrrole) nanocomposite	- 35	(*33*)

[1] Self Assembled Monolayer.

2.1. Direct Electrochemical Detection of Superoxide

The direct electrochemical detection of superoxide generated by cells was initiated by the group of Allen O. Hill. In their first paper on the issue, they report on a current signal that i.) is observed only in presence of human neutrophils, ii.) is observed only when human immunoglobulin is previously adsorbed onto the electrode, and iii.) is significantly decreased when superoxide dismutase (a superoxide scavenging enzyme) or N-ethylmaleimide (an NADPH oxidase inhibitor) is present (*18*). These properties of the current signal indicated that superoxide released by neutrophils (following their activation by the surface immobilized human immunoglobulin) was for the first time electrochemically measured. The processes (i.e. mass transport and electron transfer) leading to the superoxide-proportional current signal are schematically shown in Figure 1. The approach was also used to the measurement of the respiratory burst of single human neutrophils (*19*). This second study brings forward an advantage of using electrochemical sensors to study living cells: such sensors are relatively easily miniaturized to a size matching that of a single cell (and this allows investigating cell heterogeneity, for example).

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

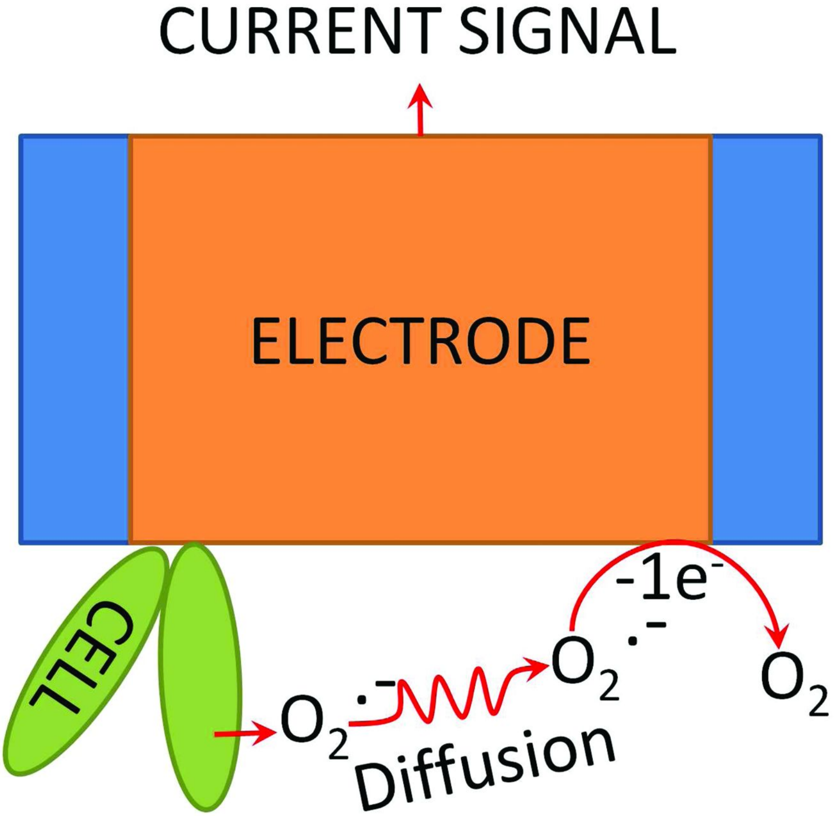


Figure 1. Electrochemical detection of the superoxide produced by cells by its direct oxidation on metal electrodes; Observations: Minimizing the distance between the source of superoxide and the interface where it is detected is very important (due to the short half-life of superoxide). Superoxide released from cells was actually for the first time electrochemically observed "collecting" human neutrophils onto opsonised electrodes (19).

Differential pulse amperometry was also used to detect superoxide by its direct oxidation on the surface of carbon fiber microelectrodes (*20*). Superoxide concentrations of 76 ± 11 nM were observed at the cell membrane level of human cord vein endothelial cells, 40 - 60 minutes after stimulation. Slightly higher concentrations of superoxide (131 ± 18 nM) were observed when using mammary artery smooth muscle cells, 1 - 2 hours after stimulation. This study highlights another advantage of using electrochemical sensors to study living cells. The same sensor can be used in conjunction with several different electrochemical methods characterized by different temporal resolution, selectivity, and sensitivity. Constant potential amperometry is most often used to get an analytical useful signal from sensors for detection of superoxide and hydrogen peroxide. In this electrochemical method the sensor is polarized at a constant potential and the current is recorded in time. The temporal resolution of the measurement can be in the millisecond domain (i.e. excellent). When using differential pulse amperometry, the sensor is again polarized at a constant potential but some potential pulses are also applied at regular intervals. The current signal will be the difference between the current prior the pulse and the current measured during the pulse. This difference will be recorded as a function of time. The temporal resolution of the method is not as good as in the case of constant potential amperometry but, by the careful selection of the pulse parameters, some additional sensitivity is gained (because contributions from baseline drift, interferences and capacitive currents are to some extent eliminated).

The group of Christian Amatore is one of the leading groups in using electrochemical microsensors (e.g. in the form of 10 μm diameter platinized carbon microelectrodes) to observe reactive oxygen and nitrogen species produced by single cells. Due to the extreme reactivity of superoxide, the group is most often not directly targeting superoxide but detecting species resulting from the disproportionation of superoxide or from the reaction of superoxide with nitric oxide instead. Quantification is carried out by direct electrooxidation of superoxide-derived species on unmodified electrodes polarized at 250 – 800 mV. When investigating the release of reactive species by interferon-gamma- or lipopolysaccharide-activated RAW 264.7 macrophages with platinized carbon microelectrodes, they have discovered that the majority of the released reactive species are likely to be derived from nitric oxide and superoxide co-produced by inducible nitric oxide synthase (*21*). Detection of reactive oxygen and nitrogen species from living cells using platinized microelectrodes integrated into microfluidic devices was also demonstrated (*22*). They have also observed that a 15 μm diameter membrane patch is activated and release reactive species when single human fibroblasts are punctured with a 1 μm diameter glass micropipette (*23*). This experimental observation is consistent with calculations which indicate that after puncturing a cell the concentration of ions, able to activate NADPH oxidase and nitric oxide synthases, significantly changes over a membrane region of about the same size. Moreover, this study demonstrates once again the excellent spatial resolution that can be achieved using electrochemical sensors to observe reactive oxygen species.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

2.2. Mediated Electrochemical Detection of Superoxide

Cytochrome c and superoxide dismutase are the two redox proteins which are most often used to build electrochemical superoxide biosensors (Table I).

Cytochrome c-based electrochemical sensors were for the first time used to detect superoxide generated by cells by the group of Calum J. McNeil (*24*). The approach is originating from the classical spectrophotometric method to detect superoxide, which uses the very fast reaction between oxidized cytochrome c and superoxide. However, in the electrochemical sensor the cytochrome c is not freely diffusing but advantageously immobilized on the electrode. Moreover, the electrode is poised to such a potential that the cytochrome c, that is reduced by superoxide, is immediately re-oxidized (by direct electron transfer) on the electrode (and thus made available for the reaction with superoxide). More superoxide will reduce more cytochrome c and will thus generate more current (Figure 2). Superoxide from human glioblastoma cells stimulated with phorbol myristate acetate or lipopolysaccharide was monitored in their first study. The cytochrome c-based sensor was positioned 1 mm away from the cells. This distance seems large taking into account the half-life of superoxide (see Introduction). However, the distance superoxide can diffuse from its source is still disputed and is clearly depending on the chemical microenvironment of the extracellular space. Detection of the superoxide from the extracellular space using cytochrome c-based sensors was later combined with the detection of superoxide in the intracellular space using fluorescence microscopy (*25*). Taking into account the problems surrounding both analytical methods, this combination, allowing the simultaneous observation of the intra- and extracellular space, is probably the most appropriate to get interesting and in the same time reliable data. The detection of superoxide using cytochrome c-based sensors was also combined with the detection of nitric oxide using a nickel tetrasulfonated phthalocyanine-based sensor (*26*). Leaving phorbol myristate acetate-activated glioblastoma cells behind, the same research group has used cytochrome-c based sensors to investigate superoxide release from UV-treated HaCaT keratinocytes (*27*). Immediate superoxide generation was observed already at relatively low doses of ultraviolet radiation. This study is interesting considering also the fact that ultraviolet A radiation was observed to compromise the effectiveness of some fluorescent probes to detect reactive oxygen species (*28*). Electrochemical sensors emerge as a good alternative to fluorescent dyes in such studies.

Cytochrome c-based electrochemical sensors were recently used to observe the time course of superoxide release by osteoclasts (bone-resorbing cells) (*29*). As a particularity, this study used simulation (of the transport and disproportionation) to estimate the magnitude of the flux of superoxide produced by the cells and to accurately determine the time-dependence of the flux in response to three different stimuli. The superoxide flux from the osteoclasts decays following a simple first-order kinetic model and much faster after stimulation with parathyroid hormone than after stimulation with vitamin D3 or pertussis toxin. Such differences suggest different mechanism of action of parathyroid hormone as compared to vitamin D3 and pertussis toxin. As already stated, such differences cannot be observed when assessing the extent of oxidative stress through endpoints.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

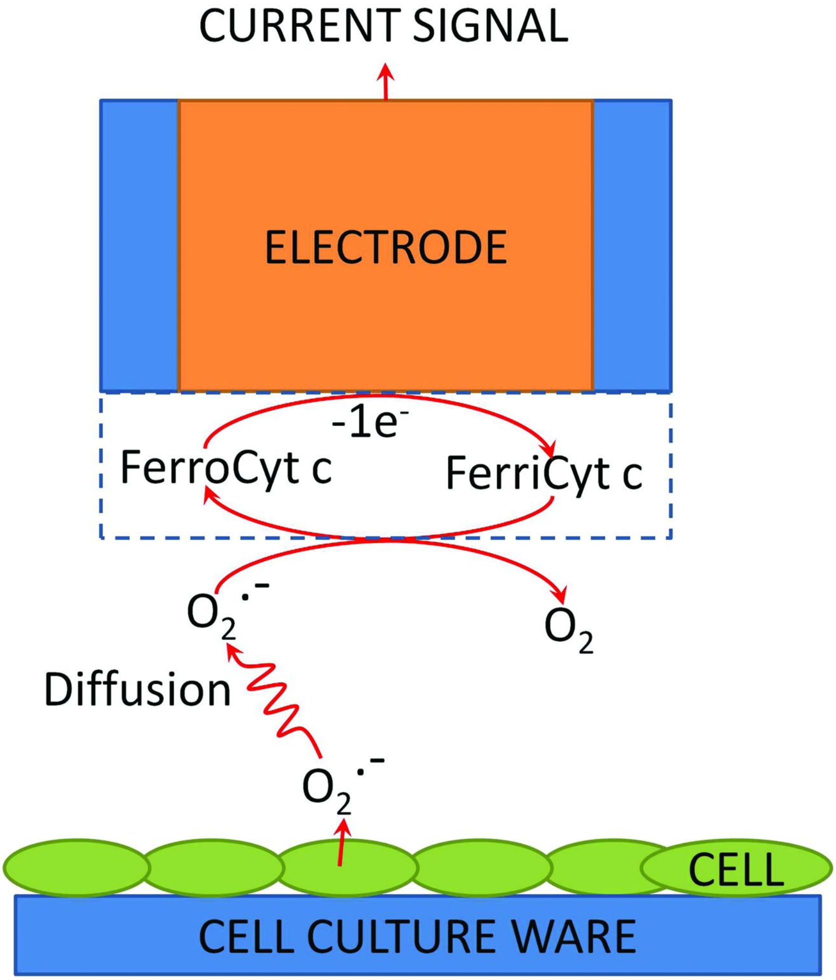


Figure 2. Electrochemical detection of the superoxide generated by cells with cytochrome c-modified electrodes; Observations: The cytochrome c heme is buried into its protein shell in a way which does not hinder direct electron transfer to the electrode. This fact allows the very fast electrochemical regeneration of the reduced cytochrome c without using any electron transfer mediator.

Our group has also used cytochrome c-modified gold electrodes to investigate the effect of calcium oxalate (one of the main components of the renal calculi) on renal cells (*30*). It was observed that A6 cells (an epithelial cell line, derived from the kidney of Xenopus laevis) release superoxide into their extracellular space in 5 - 6 minutes after encountering calcium oxalate crystals. The amount of released superoxide peaks at about 20 min. The superoxide release ceases only

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

after approximately 3 hours. This study proves that electrochemical sensors for the detection of superoxide can be useful tools also for biologists investigating the mechanism of nephrolithiasis at cellular level. Oxidative stress is accepted as being part of the pathogenesis of kidney stones (*31*) but details of the exact mechanism of stone formation and of the effect of stones on renal cells are just emerging.

Superoxide dismutase-modified electrodes will generate superoxide-proportional current signals in a very similar manner with cytochrome c-modified electrodes. Reduced and oxidized superoxide dismutase replaces ferrocytochrome c and ferricytochrome c, respectively, in the reaction pathway depicted in Figure 2. Superoxide dismutase-modified electrodes were recently used to observe the superoxide produced by activated neutrophils found in raw milk (*32*). The concept can be used to identify dairy cows suffering from subclinical mastitis based on the fact that mastitis increases the number of neutrophils in milk. This is one of the few studies indicating that detection of reactive oxygen species from cells with electrochemical sensors can be more than fundamental research. Superoxide dismutase-based superoxide sensors were also successfully used to detect superoxide from MCF-7 cancer cells (*33*). Superoxide in concentration of 0.1 ± 0.02 μM was observed 48 hours following stimulation of the cells with 50 ng mL^{-1} lipopolysaccharide. It is noteworthy that superoxide dismutase catalyzes the oxidation of superoxide to oxygen as well as its reduction to hydrogen peroxide. Therefore, this enzyme allows constructing superoxide biosensors which can be operated both at oxidation potentials and at reduction potentials. Such a flexibility can be important if interferences with the electrochemical detection are observed at a certain working potential.

Very seldom other redox proteins than cytochrome c and superoxide dismutase were also used. Pseudomonas aeruginosa azurin successfully replaced cytochrome c in biosensors for the detection of the superoxide released by human neutrophils (*34*). In addition to exploring a new biorecognition element for the electrochemical detection of superoxide, this study also promotes the simultaneous use of electrochemical and chemiluminescence-based methods to observe reactive oxygen species from living cells.

A thorough comparison of the sensors based on direct electrooxidation of superoxide with the biosensors based on redox protein-mediated electrooxidation of superoxide, although scientifically interesting and important, was not yet carried out to the best of our knowledge. However, it was already observed that part of the signal generated by the biosensors (theoretically working exclusively through mediated electrooxidation of superoxide) is actually coming from the direct oxidation of the superoxide on the electrode (*35*). This is not very surprising taking into account that the use of biorecognition elements such as cytochrome c or superoxide dismutase does not shift the working potential of the sensors to potentials lower than necessary for direct oxidation of superoxide (see Table I). However, somewhat better selectivity and better resistance to fouling of the biorecognition element-based sensors was still observed (*35*).

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

3. Electrochemical Sensors and Biosensors for Detection of Hydrogen Peroxide

Just like in the case of superoxide, there are two types of electrochemical sensors used to detect hydrogen peroxide produced by living cells (see Table II for few examples). The first type of sensor is based on the direct oxidation/reduction of hydrogen peroxide on unmodified electrode. This approach is made possible by the relatively low standard potential of the hydrogen peroxide/oxygen couple (-0.146 V vs. NHE (*13*)). The second type of sensor is based on the redox protein-mediated reduction of hydrogen peroxide on the electrode. The selectivity of the redox protein – substrate reaction is again added to the sensitivity of the electrochemical detection in such devices.

3.1. Direct Electrochemical Detection of Hydrogen Peroxide

Sensors for the detection of hydrogen peroxide based on its direct electrooxidation (Figure 3) have the advantage of simplicity and the disadvantage of being prone to interferences. However, experiments on living cell are carried out in environments which are very well controlled from chemical composition point of view (i.e. cells are bathed in solutions without compounds interfering with the electrochemical detection). Moreover, carrying out some control experiments (e.g. in the presence of catalase) is always at hand to confirm results. Therefore, detecting hydrogen peroxide by direct electrooxidation on the electrode is rather popular in the few laboratories investigating living cells with electrochemical tools.

Hydrogen peroxide, resulting from the disproportionation of the superoxide produced by the membrane-bond NADPH oxidase of neutrophils, was measured with bare carbon electrodes (*36*). Both human neutrophils and PLB 985 cells (differentiated into neutrophil-like cells) were used. Human neutrophils produced superoxide/hydrogen peroxide faster and in higher amounts. 5.5 μM of hydrogen peroxide was detected from 500000 cells suspended in 200 μL of solution. Diphenyleneiodonium chloride (an inhibitor of NADPH oxidase), superoxide dismutase (a superoxide scavenger) and catalase (a hydrogen peroxide scavenger) were all used to prove that hydrogen peroxide is selectively detected.

The hydrogen peroxide produced by human monocytic leukemia cell line THP-1, during and after differentiation into macrophages, was also monitored based on electrooxidation on bare platinum microelectrodes (*37*). 100 minutes after treating THP-1 cells with 20 nM phorbol myristate acetate the current signal corresponding to the extracellular hydrogen peroxide started to increase and remained elevated for 3 hours. Such an evolution corresponds to the time scale of the gene expression triggered by phorbol myristate acetate.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Table II. Design details of some electrochemical sensors and biosensors already used for the detection of hydrogen peroxide from cells

Electrode	*Biorecognition element*	*Immobilization method*	*Working potential / mV*	*Ref.*
100 µm diameter poly(o-phenylenediamine) covered Pt microelectrode	-	-	+ 650	(*42*)
7 µm diameter platinum disk	-	-	+ 700	(*37*)
3 mm diameter graphite disk	Horseradish peroxidase	Simple adsorption	- 50	(*34*)
TiO_2 nanoneedle-covered ITO glass	Cytochrome c	Simple adsorption	0	(*44*)
Screen printed carbon	-	-	+ 650	(*36*)
0.5 mm x 2 mm film of ZnO nanosheets	Cytochrome c	Simple adsorption	0	(*45*)
5 mm diameter ITO glass disk	Horseradish peroxidase	Os-based redox hydrogel	0	(*51*)
25 µm diameter gold disk	-	-	+ 800	(*41*)
3 mm diameter glassy carbon disk covered with hydroxyapatite nanostructures	Horseradish peroxidase	Simple adsorption	- 400	(*46*)

Scanning Electrochemical Microscopy (SECM) is a scanning probe technique that adds excellent spatial resolution to the sensitivity of electrochemical detection with microelectrodes, and which was consequently used to investigate hydrogen peroxide generation at single cell level. For example, the release of hydrogen peroxide from macrophage cells (RAW264.7) was recently investigated with SECM by Z. Ding and coworkers (*38*). A microelectrode, polarized at superimposed AC and DC potentials, was scanned above the cells at a well-controlled and constant distance (8.5 µm). The system allowed visualization of both topography and physiological activity of single cells. It was discovered that the nucleus region of the cell releases more ROS than other organelle regions. The hydrogen peroxide concentration gradient from the extracellular space of T24 human bladder cells (exposed or not to heat-killed uropathogenic Escherichia coli GR-12) was also visualized by the same group (*39*, *40*). As another particularity of their investigations, large negative DC potentials are applied onto the microelectrodes (e.g. -800 mV) instead of the more commonly used oxidation potentials. Both dissolved oxygen and hydrogen peroxide is reduced on the electrode surface at such potentials. Moreover, oxygen cannot be removed from the solution (e.g. by passing nitrogen through the systems) due to the aerobic nature of the cells (which die in the absence of dissolved oxygen). However, oxygen reduction can be discriminated from that of hydrogen peroxide by means of the SECM (*39*).

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

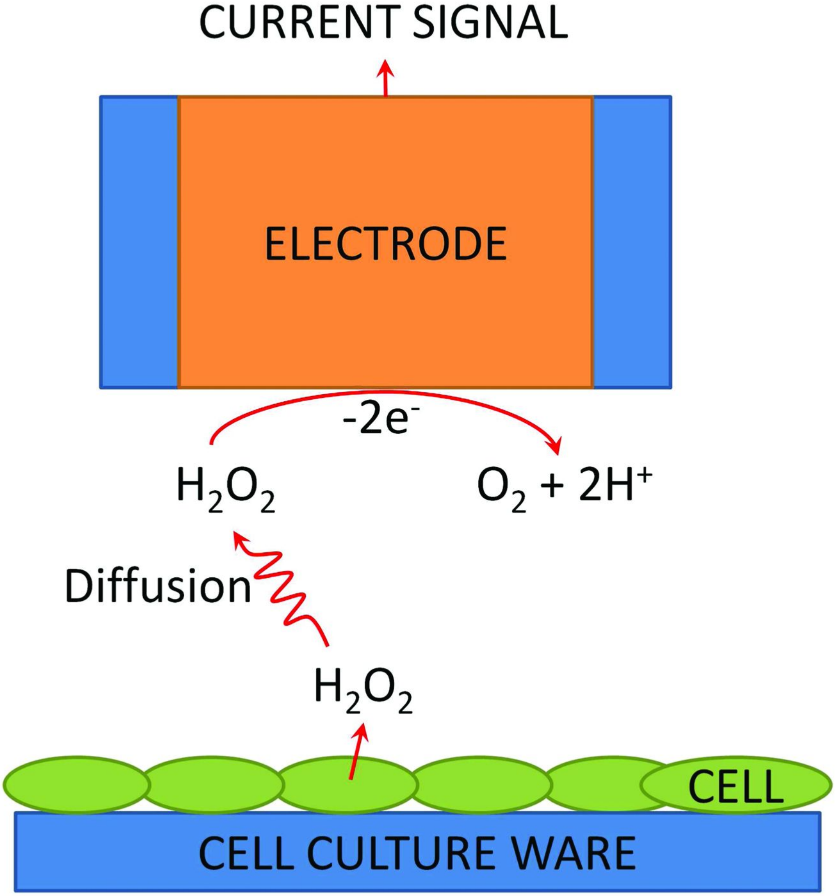


Figure 3. Electrochemical detection of the hydrogen peroxide produced by cells by its direct oxidation on metal electrodes; Observations: Sensors covered with permselective membranes (such as poly(phenylenediamine) will function based on the very same electrochemical reaction. If hydrogen peroxide is directly generated by cells or appears by the disproportionation of superoxide must be revealed using scavengers and inhibitors.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

SECM was also used to map the hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm made of *Streptococcus gordonii* and *Aggregatibacter actinomycetemcomitans* (two microbes which can coexist in the oral cavity) (*41*). Hydrogen peroxide in concentrations as high as 1.4 mM was observed 100 µm away from the *Streptococcus gordonii* films. The 1mm diameter spots of *Aggregatibacter actinomycetemcomitans* made in such films were found to consume about 66 % of the hydrogen peroxide from their close proximity by using KatA catalase.

Modifying the surface of the electrode with a permselective membrane is another approach to assure better selectivity in the electrochemical detection of hydrogen peroxide produced by cells. The porous structure of the permselective membrane will let small molecules (such as hydrogen peroxide) pass through but will repel larger molecules (e.g. ascorbate, another easily electrooxidizable compound). An example of such permselective membrane is poly(phenylenediamine). This polymer can be easily and reproducibly deposited onto the electrode by electropolymerization, and was several times proved to assure excellent selectivity to electrochemical sensors for hydrogen peroxide. Platinum microelectrodes covered with poly(o-phenylenediamine) were used to detect hydrogen peroxide generated by the NADPH oxidase of human polymorphonuclear leukocytes (*42*). After activation of the leukocytes with phorbol myristate acetate, the hydrogen peroxide concentration increased up to 5 – 15 µM, within 3 to 8 min, and then decreased slowly. Together with other similar studies, this study brings forward another advantage of using electrochemical sensors to detect reactive oxygen species in the extracellular space of living cells: the analytical signal of such devices is generated by perfectly reversible processes. Therefore, such devices will allow monitoring not only release but also consumption of reactive oxygen species by cells. In another example, the degradation of hydrogen peroxide by wild-type cells and by catalase-overexpressing mouse embryonic fibroblasts was monitored using reduction of hydrogen peroxide on a mercury film working electrode (*43*)

Detection of hydrogen peroxide on bare electrodes requires rather high applied potentials. Unfortunately, several small molecules, very often found in biological systems, are also oxidized/reduced at such high potentials. Therefore, in order to avoid selectivity problems, electrodes are often modified with a biocatalyst. This biocatalyst first of all selectively converts hydrogen peroxide and then it can be electrochemically regenerated at lower applied potential than necessary for the direct oxidation/reduction of hydrogen peroxide.

3.2. Mediated Electrochemical Detection of Hydrogen Peroxide

Unlike in the case of superoxide, where the direct electrooxidation occurs at about the same potentials as the mediated electrooxidation, the use of biocatalyst significantly lowers the potential necessary for the detection of hydrogen peroxide (as shown also in Table II). For example, when using horseradish peroxidase as biorecognition element, the detection of hydrogen peroxide actually switches from the commonly used oxidation to reduction. As such, the mediated electrochemical detection really provides a way to decrease problems related to interferences from easily oxidizable compounds. Cytochrome c and horseradish peroxidase are two biocatalysts which are often used to construct sensors for hydrogen peroxide detection. The working potential is lowered with hundreds of millivolts.

The use of cytochrome c-modified electrodes to detect hydrogen peroxide released from cells might be surprising taking into account that this redox protein was just described as a good recognition element for superoxide (and also the co-existence of the two reactive species in biological systems). As a matter of fact, oxidized cytochrome c (ferricytochrome c) catalyzes the oxidation of superoxide while reduced cytochrome c (ferrocytochrome c) catalyzes the reduction of hydrogen peroxide. Therefore, a cytochrome c-modified electrode will allow detection of both superoxide (at positive applied potentials such as + 100 mV) and hydrogen peroxide (at negative applied potentials such as -50 mV). If the rate of the heterogeneous electron transfer (e.g. that involved in the reduction of oxidized cytochrome c) is kept sufficiently high by the applied overpotential, interferences from unwanted chemical reactions (e.g. reduction of oxidized cytochrome c by superoxide from solution) are avoided. The possibility to control the selectivity of the sensor by the applied potential is one of the great advantages of the electrochemical sensors. Cytochrome c-modified TiO_2 nanoneedles were used to detect hydrogen peroxide generated by liver cancer cells (Hep G2) following stimulation with phorbol myristate acetate (*44*). In a more recent study by the same authors, ZnO nanosheets replace the TiO_2 nanoneedles and human hepatoma cells the liver cancer cells (*45*). These two studies highlight the fact that metal oxide nanostructures can serve as advantageous supports for immobilization of redox proteins. The formal potential of cytochrome c is shifted to values (108 mV and 338 mV versus Ag/AgCl when using TiO_2 and ZnO nanostructures, respectively) which allow detection of hydrogen peroxide without anodic or cathodic interferences (such as ascorbic acid and oxygen, respectively).

Horseradish peroxidase is a more traditional catalyst for reduction of hydrogen peroxide as it is widely used as label in different bioanalytical assays (e.g. ELISA). Horseradish peroxidase-modified graphite electrodes were used to detect hydrogen peroxide generated by neutrophils activated with formyl-methionine-leucine-phenylalanine peptide (*34*). The bioelectrochemical process producing the hydrogen peroxide-proportional signal is schematically depicted in Figure 4. Diphenyleneiodonium chloride and catalase were again used to prove that hydrogen peroxide generated via NADPH oxidase is indeed detected.

More recently, horseradish peroxidase was absorbed onto hydroxyapatite nanostructures (based on the electrostatic attraction between hydroxyapatite and horseradish peroxidase) and the resulting nanohybrids were further adsorbed onto the surface of a glassy carbon electrode (*46*). The resulting sensor was detecting hydrogen peroxide at -400 mV with no interferences from uric acid, ascorbic acid, glucose and 3,4-dihydroxyphenylacetic acid, and was also used to detect hydrogen peroxide from within (or released by) RAW 264.7 macrophage cells. The hydrogen peroxide concentration inside unstimulated cells was found to be 8.0 ± 0.4 nM. Whether variations in the concentration of the dissolved oxygen affect the current signal of the sensor or not remained unclear. One can expect interferences from the reduction of dissolved oxygen at working potentials as

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

negative as -400 mV. The authors used catalase to prove that recorded signals are due to hydrogen peroxide in their proof of principle experiments. Attapulgite, instead of hydroxyapatite, was also used to build sensors which proved suitable to observe hydrogen peroxide production by RAW 264.7 macrophage cells (*47*).

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

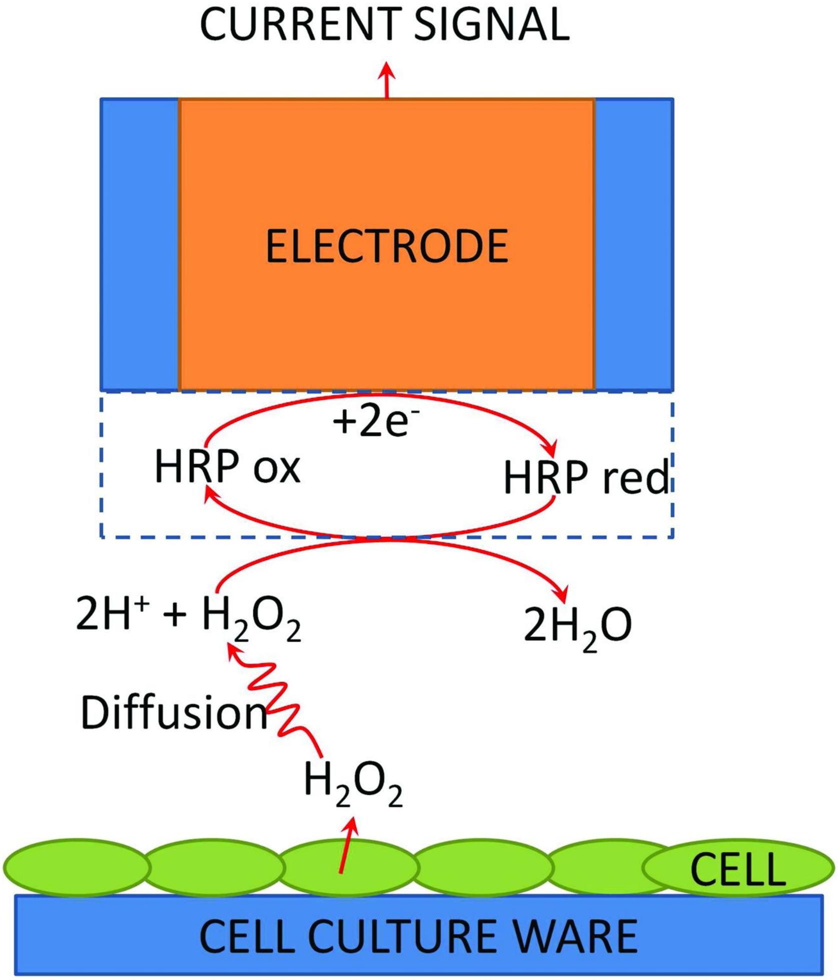


Figure 4. Electrochemical detection of the hydrogen peroxide generated by cells with horseradish peroxidase-modified electrodes. Observations: The horseradish peroxidase redox center is buried into its protein shell in a way which does not hinder direct electron transfer from the electrode. This fact allows the very fast electrochemical regeneration of the oxidized horseradish peroxidase without using any electron transfer mediator.

Hydrogen peroxide sensors, based on direct electron transfer between horseradish peroxidase and an electrode, are often characterized by small current signals. The small number of electroactive enzyme molecules and the small rate of the heterogeneous electron transfer are among the main reasons of this situation. Heterogeneous electron transfer rates are small due to the insulating protein shell (surrounding the redox center of the enzyme and thus increasing the distance between the redox center of the enzyme and the electrode) (*48*). The number of electroactive enzyme molecules is small because only molecules which are in direct contact with the electrode (i.e. in the first monolayer) and which are also correctly oriented are able of direct electron transfer to/from the electrode (*49*). As shown above, using nanostructures (to tremendously increase the surface area of the electrode and thus the number of electroactive enzyme molecules) is one way of increasing current signals in such sensors. Another interesting way is mediating the electron transfer (and thus increasing electron transfer rates) between horseradish peroxidase and electrode as well. Using redox hydrogels is one of the most efficient ways of mediating electron transfer from/to enzyme molecules (*50*). Redox hydrogels will immobilize the enzyme onto the electrode surface and in the same time will provide a network of small redox moieties (e.g. $Os^{2+/3+}$ complexes) which will shuttle electrons between the enzyme and electrode at increased rates. The use of a sensor with such an advanced design to detect hydrogen peroxide from leukocytes was recently described (*51*). Granulocyte-like differentiated HL-60 cells were stimulated with phorbol myristate acetate to release hydrogen peroxide. The hydrogen peroxide production rates (from 10 to 30 minutes after stimulation with phorbol myristate acetate) were 6, 18 and 4 pmol min^{-1} per 1×10^5 cells without any scavenger, with superoxide dismutase, and with catalase, respectively. Apocynin (another inhibitor of superoxide release from NADPH oxidase) and catalase reduced the current signal while SOD enhanced it, indicating thus NADPH oxidase as the source of the observed hydrogen peroxide. When comparing results obtained with the electrochemical hydrogen peroxide sensor and results obtained with luminol- and horseradish peroxidase-based chemiluminescence, the authors observed that the optical method detects first of all superoxide.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

4. Comparison with Common Optical Methods To Detect Superoxide and Hydrogen Peroxide at Cellular Level

Using fluorescent probes is a very popular choice when it comes to the detection of reactive oxygen species in/from living cells. The fluorescent probes used to quantify reactive oxygen species in cells have recently been reviewed elsewhere (*52*, *53*). However, several papers revealing lack of selectivity of some of the probes have also been published. It was reported that even Dulbecco’s Modified Eagle’s Medium confer fluorescence intensity changes in dihydrorhodamine 123 and 2′,7′-dichlorodihydrofluorescein diacetate (two commercially available and popular probes) and that pre-treatment with ultraviolet A radiation compromises the effectiveness of these probes to detect ROS generated in a cell-free system (*28*). When examining the ability of

2′,7′-dichlorofluorescein, dihydroethidine, dihydrorhodamine, 3′-(p aminophenyl) fluorescein, and 4′,5′-diaminofluorescein to detect reactive oxygen and nitrogen species in cell-free systems, it was observed that only 3′-(p aminophenyl) fluorescein demonstrates a high degree of specificity for a single reactive species (*54*).

In order to improve the fluorescence-based detection of reactive oxygen species two strategies are usually followed. The first strategy is to synthesize new dyes, while the second is to build on advantages of nanotechnology. Several new dyes, such as bis(p-methylbenzenesulfonate)dichlorofluorescein (*55*), and naphtho-peroxyfluor-1 (*56*) were recently used to selectively detect hydrogen peroxide in cells. Following the second strategy, nanoencapsulation was used to improve the selectivity for hydrogen peroxide of a traditional fluorescent probe (2′,7′-dichlorofluorescin) (*57*), and fluorescent single-walled carbon nanotubes were used to monitor hydrogen peroxide released from individual A431 human epidermal carcinoma cells (*8*). These nanotechnology-based approaches offer also excellent spatial and temporal resolution.

Chemiluminescent probes are also used to detect reactive oxygen species. A recent study comparing twenty chemiluminescent probes identified nine probes having selectivity for superoxide, and one probe with selectivity for hydroxyl radicals. No chemiluminescent reagent was found to be selective for hydrogen peroxide, hypochlorite anion, singlet oxygen and nitric oxide (*58*). Another study has discovered that annexin AI-derived peptides reduce the light output with isoluminol excited by superoxide and horseradish peroxidase in formyl-methionyl-leucyl-phenylalanine- and phorbol myristate acetate-stimulated cells (as well as by hydrogen peroxide and HRP). Therefore, a reduced cellular response detected with isoluminol-amplified chemiluminescence should be confirmed with an alternative technique to determine release of superoxide anions and hydrogen peroxide (*59*).

In a comparative investigation, fluorometric, colorimetric, and chemiluminometric methods were applied to determine ROS production by NADPH oxidases expressed by human lung epithelial cell line A549. The fluorometric methods were based on 2′,7′-dichlorofluorescein-diacetate, dihydroethidium, or amplex red, the colorimetric methods were based on nitrobluetetrazolium or cytochrome c, while the chemiluminometric methods were luminol- and L-012-amplified. The fluorometric methods were found to be the most suitable for the purpose (*60*).

Taken all these together one can conclude that, although used much more widely than electrochemical sensors to detect reactive oxygen species in cell biology, methods based on optical probes are far from perfect, first of all, regarding their selectivity and their ability to offer temporal resolution. Moreover, the majority of the probes are used to investigate the intracellular space. Although reactive oxygen species are generated inside the cell (and thus monitoring the intracellular space makes perfect sense), in order to act as intercellular signaling molecules, reactive oxygen species must leave the intracellular space and be able to travel to neighboring cells. Monitoring reactive oxygen species in the extracellular space with electrochemical sensors makes therefore also sense. Unlike the optical methods, electrochemical sensors for the detection of reactive

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

oxygen species do not require addition of a dye to the cells. Therefore, cells will be minimally disturbed during electrochemical investigations. Electrochemical sensors can also be calibrated just before their use on cellular models using enzymatically (e.g. with xanthine oxidase and xanthine) or chemically (e.g. with potassium superoxide) produced superoxide, and conditions similar to those of the experiment on cellular models. Obtaining quantitative information on the absolute concentration of the reactive oxygen species is thus somewhat easier using electrochemical sensors than optical probes. Last but not least, unlike most of the optical probes which will suffer irreversible chemical changes in their interaction with reactive oxygen species, electrochemical sensors are reversible, that is, they can monitor both generation and consumption of reactive oxygen species by cells.

5. Conclusions

When carefully designed, constructed and then positioned above cells, electrochemical sensors and biosensors can continuously monitor the appearance, and the subsequent disappearance, of superoxide and hydrogen peroxide in the extracellular space. The continuous monitoring allows observing the dynamics of the process. Differences in the dynamics of the process can be indicators of different mechanism of action which are difficult to observe with analytical methods providing only endpoints (e.g. hydrogen peroxide concentration at the end of few hours long incubation). However, electrochemical sensors able to detect superoxide and hydrogen peroxide were relatively seldom used to investigate living cells. Their popularity among cell biologists is very far from the popularity of fluorescent and chemiluminescent probes. As a consequence, their reliability in such applications is still to be proved. The use of specific scavengers (such as catalase and superoxide dismutase) and of specific inhibitors (such as inhibitors of NADPH oxidase or of the mitochondrial respiration) is recommended in order to overcome any selectivity problems.

There are several interesting questions surrounding the oxidative stress in living cells. Where the superoxide/hydrogen peroxide observed in the extracellular space come from? Some published papers sustain that mitochondrial reactive oxygen species are released into the cytoplasm, but they do not escape from the cell (*61*). Therefore, the reactive oxygen species electrochemically observed in the extracellular space could be generated by membrane bound enzymes (such as NADPH oxidase) rather than by mitochondria. Alternatively, they could be still of mitochondrial origin and could escape from the cell due to membrane poration in the given experimental conditions. Is superoxide, hydrogen peroxide or both causing oxidative stress and/or signaling to neighboring cells? There are several facts making this question difficult to answer. The fast dismutation of superoxide giving hydrogen peroxide is one of these facts. Some NADPH oxidases (Nox1 and Nox 2) generate superoxide while other NADPH oxidases (Nox 4) generate hydrogen peroxide due to the structure of their E-loop (*62*), is another fact. Moreover, several widely used inhibitors of NADPH oxidase (e.g. diphenylene iodonium and apocynin) were found to be not specific and artifact free complicating the situation (*63*, *64*). The simultaneous optical and electrochemical monitoring of the cells (to simultaneously observe reactive oxygen species in the intracellular space and in the extracellular space, respectively), seems to be a very promising approach to answer these interesting question. However one should take into account that optical probes (just as the electrochemical sensors) function while consuming reactive oxygen species. As such, they will decrease the chances of these species reaching the extracellular space.

References

1. Tlili, A.; Dupre-Crochet, S.; Erard, M.; Nusse, O. *Free Radical Biol. Med.* **2011**, *50*, 438–447.
2. Ardanaz, N.; Pagano, P. J. *Exp. Biol. Med.* **2006**, *231*, 237–251.
3. Niethammer, P.; Grabher, C.; Look, A. T.; Mitchison, T. J. *Nature* **2009**, *459*, 996–U123.
4. Freitas, M.; Porto, G.; Lima, J.; Fernandes, E. *Biometals* **2010**, *23*, 31–41.
5. Grammas, P.; Martinez, J.; Miller, B. *Expert Rev. Mol. Med.* **2011**, *13*, 22.
6. Manea, A. *Cell Tissue Res.* **2011**, *342*, 325–339.
7. Saran, M.; Bors, W. *Chem.-Biol. Interact.* **1994**, *90*, 35–45.
8. Jin, H.; Heller, D. A.; Kalbacova, M.; Kim, J. H.; Zhang, J. Q.; Boghossian, A. A.; Maheshri, N.; Strano, M. S. *Nat. Nanotechnol.* **2010**, *5*, 302–U81.
9. Barnard, M. L.; Matalon, S. *J. Appl. Physiol.* **1992**, *72*, 1724–9.
10. Dringen, R.; Hamprecht, B. *Brain Res.* **1997**, *759*, 67–75.
11. Li, H. M.; Li, O. L.; Wang, X.; Xu, K. H. H.; Chen, Z. Z.; Gong, X. C.; Liu, X.; Tong, L. L.; Tang, B. *Anal. Chem.* **2009**, *81*, 2193–2198.
12. Karlsson, A.; Nixon, J. B.; McPhail, L. C. *J. Leukocyte Biol.* **2000**, *67*, 396–404.
13. Amatore, C.; Arbault, S. Oxidative Stress at the Single Cell Level. In *Electrochemical Methods for Neuroscience*; Michael, A. C., Borland, L. M., Eds.; CRC Press: Boca Raton, FL, 2007.
14. Amatore, C.; Arbault, S.; Guille, M.; Lemaitre, F. *Chem. Rev.* **2008**, *108*, 2585–2621.
15. Yuasa, M.; Oyaizu, K. *Curr. Org. Chem.* **2005**, *9*, 1685–1697.
16. Bedioui, F.; Quinton, D.; Griveau, S.; Nyokong, T. *Phys. Chem. Chem. Phys.* **2010**, *12*, 9976–9988.
17. Petlicki, J.; van de Ven, T. G. M. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 2763–2767.
18. Green, M. J.; Hill, H. A. O.; Tew, D. G.; Walton, N. J. *FEBS Lett.* **1984**, *170*, 69–72.
19. Hill, H. A. O.; Tew, D. G.; Walton, N. J. *FEBS Lett.* **1985**, *191*, 257–263.
20. Privat, C.; Stepien, O.; David-Dufilho, M.; Brunet, A.; Bedioui, F.; Marche, P.; Devynck, J.; Devynck, M. A. *Free Radical Biol. Med.* **1999**, *27*, 554–559.
21. Amatore, C.; Arbault, S.; Bouton, C.; Drapier, J. C.; Ghandour, H.; Koh, A. C. W. *ChemBioChem* **2008**, *9*, 1472–1480.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

22. Amatore, C.; Arbault, S.; Chen, Y.; Crozatier, C.; Tapsoba, I. *Lab-on-a-Chip* **2007**, *7*, 233–238.
23. Amatore, C.; Arbault, S.; Erard, M. *Anal. Chem.* **2008**, *80*, 9635–9641.
24. Manning, P.; McNeil, C. J.; Cooper, J. M.; Hillhouse, E. W. *Free Radical Biol. Med.* **1998**, *24*, 1304–1309.
25. Chang, S. C.; Rodrigues, N. P.; Zurgil, N.; Henderson, J. R.; Bedioui, F.; McNell, C. J.; Deutsch, M. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 979–984.
26. Chang, S. C.; Pereira-Rodrigues, N.; Henderson, J. R.; Cole, A.; Bedioui, F.; McNeil, C. J. *Biosens. Bioelectron.* **2005**, *21*, 917–922.
27. Aitken, G. R.; Henderson, J. R.; Chang, S. C.; McNeil, C. J.; Birch-Machin, M. A. *Clin. Exp. Dermatol.* **2007**, *32*, 722–727.
28. Boulton, S.; Anderson, A.; Swalwell, H.; Henderson, J. R.; Manning, P.; Birch-Machin, M. A. *Free Radical Res.* **2011**, *45*, 115–122.
29. Berger, C. E. M.; Dattaa, H. K.; Horrocks, B. R. *Phys. Chem. Chem. Phys.* **2011**, *13*, 5288–5297.
30. Gáspár, S.; Niculite, C.; Cucu, D.; Marcu, I. *Biosens. Bioelectron.* **2010**, *25*, 1729–1734.
31. Escobar, C.; Byer, K. J.; Khaskheli, H.; Khan, S. R. *J. Urol.* **2008**, *180*, 379–387.
32. Okada, K.; Fukuda, J.; Suzuki, H.; Ayano, S.; Nikaido, Y.; Nishi, T.; Oka, K. *IEEE Sensors J.* **2009**, *1-3*, 1694–1697.
33. Rajesh, S.; Kanugula, A. K.; Bhargava, K.; Ilavazhagan, G.; Kotamraju, S.; Karunakaran, C. *Biosens. Bioelectron.* **2010**, *26*, 689–695.
34. Shleev, S.; Wettero, J.; Magnusson, K. E.; Ruzgas, T. *Cell Biol. Int.* **2008**, *32*, 1486–1496.
35. Chen, X. J.; West, A. C.; Cropek, D. M.; Banta, S. *Anal. Chem.* **2008**, *80*, 9622–9629.
36. Ashkenazi, A.; Abu-Rabeah, K.; Marks, R. S. *Talanta* **2009**, *77*, 1460–1465.
37. Kasai, S.; Shiku, H.; Torisawa, Y.-s.; Noda, H.; Yoshitake, J.; Shiraishi, T.; Yasukawa, T.; Watanabe, T.; Matsue, T.; Yoshimura, T. *Anal. Chim. Acta* **2005**, *549*, 14–19.
38. Zhao, X. C.; Diakowski, P. M.; Ding, Z. F. *Anal. Chem.* **2010**, *82*, 8371–8373.
39. Zhao, X.; Zhang, M.; Long, Y.; Ding, Z. *Can. J. Chem.* **2010**, *88*, 569–576.
40. Zhao, X.; Lam, S.; Jass, J.; Ding, Z. *Electrochem. Commun.* **2010**, *12*, 773–776.
41. Liu, X. H.; Ramsey, M. M.; Chen, X. L.; Koley, D.; Whiteley, M.; Bard, A. J. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 2668–2673.
42. Liu, X. P.; Zweier, J. L. *Free Radical Biol. Med.* **2001**, *31*, 894–901.
43. Lahdesmaki, I.; Park, Y. K.; Carroll, A. D.; Decuir, M.; Ruzicka, J. *Analyst* **2007**, *132*, 811–817.
44. Luo, Y. P.; Liu, H. Q.; Rui, Q.; Tian, Y. *Anal. Chem.* **2009**, *81*, 3035–3041.
45. Rui, Q.; Komori, K.; Tian, Y.; Liu, H. Q.; Luo, Y. P.; Sakai, Y. *Anal. Chim. Acta* **2010**, *670*, 57–62.
46. Li, C. Y.; Zhang, H.; Wu, P.; Gong, Z. N.; Xu, G. L.; Cai, C. X. *Analyst* **2011**, *136*, 1116–1123.

47. Wu, P.; Cai, Z. W.; Chen, J.; Zhang, H.; Cai, C. X. *Biosens. Bioelectron.* **2011**, *26*, 4012–4017.
48. Presnova, G.; Grigorenko, V.; Egorov, A.; Ruzgas, T.; Lindgren, A.; Gorton, L.; Borchers, T. *Faraday Discuss.* **2000**, *116*, 281–289.
49. Zimmermann, H.; Lindgren, A.; Schuhmann, W.; Gorton, L. *Chem.−Eur. J.* **2000**, *6*, 592–599.
50. Heller, A. *Curr. Opin. Chem. Biol.* **2006**, *10*, 664–672.
51. Inoue, K. Y.; Ino, K.; Shiku, H.; Kasai, S.; Yasukawa, T.; Mizutani, F.; Matsue, T. *Biosens. Bioelectron.* **2010**, *25*, 1723–1728.
52. Gomes, A.; Fernandes, E.; Lima, J. L. F. C. *J. Biochem. Biophys. Methods* **2005**, *65*, 45–80.
53. Dickinson, B. C.; Srikun, D.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2010**, *14*, 50–56.
54. Price, M.; Kessel, D. *J. Biomed. Opt.* **2010**, *15*, 3.
55. Zhang, X. Y.; Li, Q. L.; Chen, Z. Z.; Li, H. M.; Xu, K. H.; Zhang, L. S.; Tang, B. *Lab-on-a-Chip* **2011**, *11*, 1144–1150.
56. Albers, A. E.; Dickinson, B. C.; Miller, E. W.; Chang, C. J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5948–5950.
57. Kim, G.; Lee, Y. E. K.; Xu, H.; Philbert, M. A.; Kopelman, R. *Anal. Chem.* **2010**, *82*, 2165–2169.
58. Yamaguchi, S.; Kishikawa, N.; Ohyama, K.; Ohba, Y.; Kohno, M.; Masuda, T.; Takadate, A.; Nakashima, K.; Kuroda, N. *Anal. Chim. Acta* **2010**, *665*, 74–78.
59. Dahlberg, M.; Dahgren, C.; Helistrand, K.; Movitz, C. *Luminescence* **2008**, *23*, 139–143.
60. Kolarova, H.; Bino, L.; Pejchalova, K.; Kubala, L. *Folia Biol. (Prague, Czech Repub.)* **2010**, *56*, 211–217.
61. Kuznetsov, A. V.; Kehrer, I.; Kozlov, A. V.; Haller, M.; Redl, H.; Hermann, M.; Grimm, M.; Troppmair, J. *Anal. Bioanal. Chem.* **2011**, *400*, 2383–2390.
62. Takac, I.; Schroder, K.; Zhang, L. L.; Lardy, B.; Anilkumar, N.; Lambeth, J. D.; Shah, A. M.; Morel, F.; Brandes, R. P. *J. Biol. Chem.* **2011**, *286*, 13304–13313.
63. Heumuller, S.; Wind, S.; Barbosa-Sicard, E.; Schmidt, H.; Busse, R.; Schroder, K.; Brandes, R. P. *Hypertension* **2008**, *51*, 211–217.
64. Wind, S.; Beuerlein, K.; Eucker, T.; Muller, H.; Scheurer, P.; Armitage, M. E.; Ho, H.; Schmidt, H.; Wingler, K. *Br. J. Pharmacol.* **2010**, *161*, 885–898.

Downloaded by OHIO STATE UNIV LIBRARIES on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch010

Chapter 11

Peroxynitrite and Nitroxidative Stress: Detection Probes and Micro-Sensors. A Case of a Nanostructured Catalytic Film

Serban F. Peteu,[1,3,4] Saleem Banihani,[1] Mutha M. Gunesekera,[1]
Pubudu Peiris,[1] Oana A. Sicuia,[5] and Mekki Bayachou*,[1,2]

[1]Chemistry Department, Cleveland State University, 2399 Euclid Ave.,
Cleveland, Ohio 44115, United States
[2]Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave. NB21,
Cleveland, Ohio 44195, United States
[3]National Institute for Chemistry Research and Development,
Bucharest 300224, Romania
[4]Chemical Engineering & Materials Science, Michigan State University,
East Lansing, Michigan 48824, United States
[5]Institute for Crop Protection Research and Development,
8 Ion Ionescu dela Brad Blvd., Bucharest 013813, Romania
*E-mail: m.bayachou@csuohio.edu

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Peroxynitrite, the primary product of the reaction of superoxide ion and nitric oxide, emerged as an important species with profound biological roles. Relatively speaking, it is a new member of the nitroxidative array of reactive metabolites, and details of its actions, impact on biological systems in health and disease states are still accumulating. It has already been linked to a host of pathological conditions. At the same time, its cytoprotective roles including redox regulation of critical signaling pathways are also reported.

Assessment of peroxynitrite's deleterious versus potential protective/signaling roles strongly depends on the possibility to accurately measure and monitor its concentration. This will help build a clearer understanding of its physiological roles. However, peroxynitrite's extremely short half-life under physiological conditions and its very complex reactivity with many cellular targets create a major analytical chemistry

challenge, particularly at the single cell level. The dynamic concentration of peroxynitrite versus other reactive species generated *in situ* under various conditions modulates its role in many vital cell functions.

In this chapter, we give a brief overview of peroxynitrite biochemistry, physiology, and related therapeutic efforts to control its impact under pathological conditions. We then discuss the challenges and accomplishments in terms of major analytical methods developed for peroxynitrite's sensing up-to-date, as well as opportunities for the future.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

1. Introduction

Peroxynitrite emerged as a potent cytotoxic compound since early 1990s (*1*, *2*) after the identification of nitric oxide as the main player involved in many physiological processes including vasodilation (*3*). It turned out to be the direct culprit for much what was wrongly assigned as nitric oxide's pathology. A plethora of physiologically relevant reactions, ranging from deleterious to protective ones, is subject of extensive research. In fact, the list of reports implicating this molecule in pathologic conditions and, more recently, as potentially involved in basic signaling or ensuring protective roles, is growing (*4–10*).

Peroxynitrite (Formula: $ONOO^-$, Abbreviation: PON; PON will be mostly used in this text; however, the full name will also be used interchangeably at times when emphasis on the name is needed) is the product of the reaction of superoxide ion ($O_2^{\bullet-}$) and nitric oxide free radical ($NO^{\bullet}$) (*2*, *11–14*). Nitric oxide is generated in a two-step catalytic oxidation of L-arginine by nitric oxide synthases (NOSs), while superoxide ion can be leaked by many oxidases, by enzyme complexes of the respiratory chain associated with mitochondria, or by simple intrinsic uncoupling of endothelial NOS (eNOS), Figure 1. Proinflammatory conditions may exacerbate the levels of superoxide ion released, and thus the levels of PON formed. Nitric oxide and superoxide ion react very fast in a diffusion-controlled reaction (reported rates ~3-20 $x10^9$ $M^{-1}s^{-1}$). A reaction rate of this magnitude outperforms even the natural enzymatic sink for superoxide ion, i.e. the catalytic detoxification by superoxide dismutases. The only case where the rate of the reaction with CO_2 is challenged is under conditions of high density of target cells, which essentially reduces the diffusion distance from a given PON source (*15*). Given the relatively large diffusion range of NO (*16*, *17*), the order of magnitude reported for the rate of reaction between nitric oxide and superoxide ion guarantees the formation of $ONOO^-$ any time NO comes across a source of superoxide ion.

Peroxynitrite chemistry is strongly dependent on pH, and is further complicated by the complexity of the biological milieu and the myriad of possible cellular targets. PON anion can act as a direct oxidant of many cellular targets. At pH 7.4, 80% of $ONOO^-$ is present as the anionic form while the complementary fraction is protonated in the form of peroxynitrous acid (ONOOH, pKa = 6.8).

The anionic form can react with many targets. For instance it reacts with CO_2 to form nitrosoperoxycarbonate adduct ($ONOOCO_2^-$), Figure 1. The O-O bond homolysis in both ONOOH (the conjugate acid of PON) and $ONOOCO_2^-$ generates deleterious radicals ($^{\bullet}OH$, $^{\bullet}NO_2$, $CO_3^{\bullet -}$), which highlights another indirect deleterious *torrent* that drives peroxynitrite's pathological impacts (*2*, *12*, *13*, *17*, *18*) (Figure 1). Hydroxyl radicals (•OH), nitrogen dioxide ($\bullet NO_2$) and carbonate radicals are behind irreversible insults on cell components such as disruption of membrane lipids, nucleobase oxidation/nitration and DNA strand breaks, and protein nitrations (*19–21*). Given its direct and indirect decay pathways, PON is relatively short-lived (<1 s) (*13*, *16*, *18*). Despite its short physiological half-life, PON may still affect cells within an area of ~2.5-10 μm radius (*18*). Although, under certain conditions, steady-state levels of peroxynitrite were reported at low nanomolar concentrations, the flux can be sustained over hours (*22*, *23*).

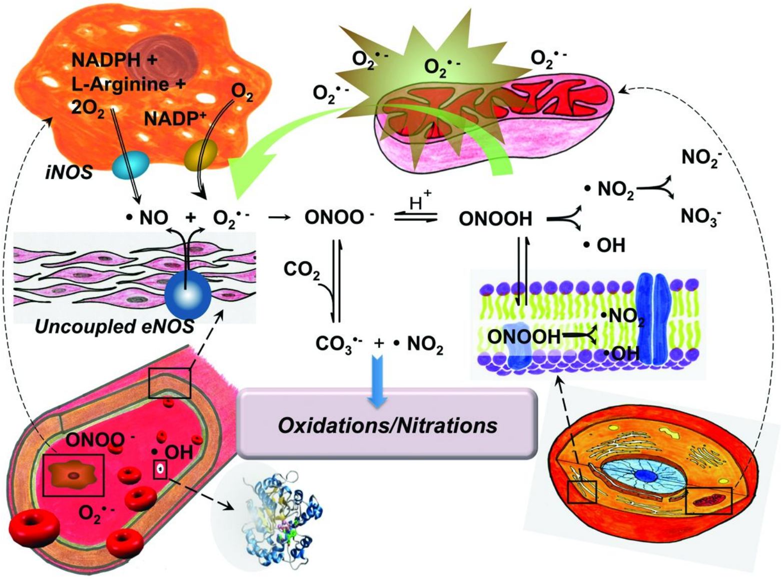


Figure 1. Major pathways of peroxynitrite generation, its direct cellular targets, and indirect radical pathways that ultimately result in oxidation, nitration, and disruption of vital cellular functions. (see color insert)

Reports on the physiological actions of PON linked this potent oxidant species to pathological conditions but also to cytoprotective roles, including redox regulation of critical signaling pathways, triggering protective signals against ischemia-reperfusion injury and neural apoptosis (*13*). The dynamic concentration of PON generated in tissues under various conditions, and the balance to local concentrations of other species such as NO, seem to modulate its role in many vital cell functions. Assessment of PON's deleterious versus potential protective/signaling roles strongly depends on the possibility to accurately measure and monitor its concentration. This will help build a clearer understanding of its physiological roles.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

In this chapter, we give an overall review that outlines the challenges and accomplishments in terms of the major methods developed for PON's sensing up-to-date. First we give a brief overview of PON biochemistry, physiology, and related therapeutics. These areas will highlight the pressing need for methods able to directly detect PON and accurately monitor its concentration under various settings.

2. Peroxynitrite's Biochemistry, Physiology, and Therapeutics: Analytical Challenges

As we briefly outlined in the introductory section, PON emerged as an important species affecting many aspects of biological systems. Below is a brief summary of how this reactive species impacts many cellular events and its induced toxicity.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

2.1. Peroxynitrite Biochemical Mechanisms Inducing Toxicity in Animals

PON formation under oxidative stress is linked to a number of assaults on cellular constituents. This reactive molecule targets many biomolecules through direct oxidation or through homolysis-derived radicals as outlined in the introduction. Substantial oxidations and other transformations of proteins, DNA, and lipids contribute to the disruption of key cellular functions. The situation can ultimately result in the induction of cell death through both apoptosis and necrosis pathways.

Cellular membranes are important targets of the molecular damage that PON triggers. PON is known to permeate lipid bilayers as peroxynitrous acid (*17*). The breakdown of this oxidant into reactive radicals triggers the peroxidation and the nitration of lipids (*24*).

On another front, PON may attack antioxidant enzymes such as superoxide dismutase and glutathione reductase, as well as, other antioxidant defense mechanisms like glutathione and cysteine (*25–30*). Manganese superoxide dismutase is one target of PON in the enzymatic antioxidant pool. Nitration of this enzyme results in loss of its optimum activity and accumulation of more superoxide ions (*26*), and thus formation of more PON. This process acts as a positive-feedback loop that exacerbate the nitroxidative stress. Similar feedback loops can result in other secondary processes. For instance, oxidation of cofactors essential to enzymatic functions is another ramp that amplifies the oxidative assault. An example is the oxidation of tetrahydropterin cofacor of nitric oxide synthase. This process limits the availability of the reduced form of the pterin, which is needed for proper enzyme function (*31*). This situation, in turn, results in NOS kinetic uncoupling and generation of more superoxide ions, which feed on NO to make more PON.

Tyrosine nitration in enzymes leads in many cases to disruption of structure and decreased function or total inactivation. In addition to the case of superoxide dismutase discussed above, prostacyclin synthase is another example of enzymes targeted by nitration (*32*) under nitroxidative stress. Other nitration targets by peroxynitrite include: channels, calcium pumps (SERCA) (*12*, *33*), tyrosine hydroxylase (*34*), epidermal growth factor receptor (EGFR) (*29*, *35*), and alpha-synuclein which leads to intracellular aggregates (*29*, *36*), to cite a few. Covalent tyrosine modifications carried by PON affect not only structure or function at the level of an individual protein, but also major cellular events such as signal transduction involving tyrosine phosphorylation.

Oxidation of cysteine residues in proteins and enzymes (*37–41*) is another outcome of cell exposure to PON, which can affect vital cellular functions.

Lastly, PON and its derived radicals can attack DNA. The assault can result in DNA base oxidation and/or nitration (*21*), or attack on deoxyribose sugar to lead to double and single strand breaks (*29*, *42*).

All of the disruptions caused by PON or its derived radicals transcend the molecular level to the subscellular level, where the cytotoxic effects become apparent. For instance, inactivation of mitochondrial superoxide dismutase upon nitration may lead to further damage such as nitration of cytochrome c, and the overall disruption of the electron transfer chain, which will ultimately yield to failure of the mitochondrion. In parallel, the nitroxidative stress affects the mitochondrion's permeability, which leads to cytochrome c release, and activation of the apoptosis route. Likewise, excessive attack on DNA activates the poly(ADP-ribose) polymerase (PARP) pathway in the nucleus (*43–45*), which ultimately commits the cell to necrosis.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

2.2. Therapeutic Approaches To Mitigate the Toxic Effects of Peroxynitrite

Endogenous Scavengers?

Evidence for the cytotoxicity of peroxynitrite and its roles in oxidative stress is continuously growing. In the previous section we outlined some aspects of peroxynitrite's impacts on cellular biochemistry (*vide supra*) (*12*, *13*, *46*). In the face of this extremely reactive oxidant, one would think that natural defense mechanisms using endogenous species must have evolved to "neutralize" its oxidative/nitrative stress. This may be the case at the level of prevention (efficient control of superoxide leaks) and/or at the level of repair (clearing of nitrated proteins/enzymes or repair of DNA, etc.), but apparently not the case at the level of direct scavenging. In fact, even though endogenous molecules such as ascorbate and glutathione can react with PON, their reaction rates are too slow to directly block peroxynitrite-induced cytotoxicity (*47*). Urate is another endogenous candidate for peroxynitrite scavenging. However, the direct reaction of urate with peroxynitrite (in the peroxynitrous acid form) is too slow ($k \sim 450$ $M^{-1}s^{-1}$) (*48*) to compete for instance with its reaction with CO_2. Yet, reports have shown that urate exhibits *some* efficiency –although limited- of inhibiting some PON-mediated processes. This inhibition, however, operates mainly via reaction of urate with secondary radicals that derive from PON (*49*).

Peroxiredoxins are widespread members of the peroxidase family that exhibit efficient, thiol-based, peroxynitrite reductase activity (observed rates $\sim 10^6 M^{-1}s^{-1}$ - $7x10^7 M^{-1}s^{-1}$). With rates of this order of magnitude, peroxiredoxins emerged as potential candidates for the *long-illusive* biological pathway of PON detoxification, and also provided a rationale for the mechanism of defense of resistant pathogens against peroxynitrite of the host immune system (*50*, *51*). However, peroxiredoxins' efficiency in neutralizing the PON threat in cells requires sufficiently high intracellular concentrations of this enzyme and a flux of peroxynitrite formation that does not overwhelm the reducing machinery of a cell to regenerate it.

Exogenous Scavengers: Mitigation Efforts against Peroxynitrite's Toxicity

On the exogenous side of compounds that can be used in the detoxification of peroxynitrite, metalloporphyrins were among the early candidates considered (*47*, *52*, *53*). Their potential use as therapeutic decomposition catalysts for peroxynitrite was studied in a broad array of nitroxidative stress-driven pathologies.

There has been constant efforts to identify compounds capable of reacting with peroxynitrite fast enough, to transform it before its reaction with cellular components (*54*, *55*). Several such agents have been reported to remove PON and reduce its injurious effects in a variety of diseases or disease models (*56*–*60*).

More recently, Tao et al. reported that a new, second generation peroxynitrite decomposition catalyst, [5,10,15,20-tetra[N-(benzyl-4-carboxylate)-2-pyridinium]-21*H*,23*H*-porphine iron(III) chloride] (INO-4885), protects the heart against reperfusion injury in a mouse model (*61*). INO-4885 appears to work 10 times faster than FP-15 analogue, an early first generation PON decomposition catalyst (*62*). The catalytic efficiency of INO-4885 allows it to be used at very low therapeutic doses. Along with iron, compounds of manganese porphyrins have also had their share in assessing their use as therapeutics against peroxynitrite molecular assaults. The list of published reports is long (*18*), but very recently, Nin *et al.* tested the performance of the catalyst manganese tetrakis(4-N-methylpyridyl)porphyrin (MnTMPyP) in scavenging PON, and found that this catalyst protects from macrovascular and microvascular dysfunction in sepsis (*63*).

The quest for efficient synthetic catalysts for the decomposition/isomerization of peroxynitrite *in vivo* will likely to continue. This may expand beyond porphyrins to other biomimetic compounds. Accurate and specific high throughput reporting probes and microsensors able to follow the fate of peroxynitrite in biological test systems will be an important part of this development, and will allow quick scanning of catalyst libraries in the search for optimal decomposition kinetics.

2.3. Peroxynitrite in Plant Response to Pathogens

Peroxynitrite Formation in Plant Organisms

The idea that PON is also generated in plant cells, was hypothesized after revealing that NO cooperates with reactive oxygen species (ROS) during plant response to biotic stress (*64*, *65*). Despite the studies carried out by Alamillo

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

and García-Olmedo (*66*, *67*) and others (*68–71*), $ONOO^-$ generation in plants remained a speculation. However, recent studies seem to provide evidence for PON formation in plant cells as well as its implications in plant physiology, such as the plant fight against pathogens (*72*). More specifically, intracellular $ONOO^-$ production was detected using bioimaging with aminophenyl fluorescein by Saito et al. (*73*) in stimulated tobacco BY-2 cells.

Because $ONOO^-$ formation depends on NO and superoxide ion co-generation, tracking their formation in plants cell can suggest the places where $ONOO^-$ may be formed. In plants, the production of superoxide anion is regulated by the *rboh* gene (*74*, *75*), while nitric oxide is produced by nitric oxide synthase (NOS) (*76*, *77*), nitrate reductase (NR) (*78*, *79*) and nitrite/nitric oxide-oxidoreductase (NI-NOR), as well as non-enzymatic mechanisms such as the decomposition of nitrous acid to NO (*79*, *80*) or the carotenoid-mediated NO production (*81*). Besides, NO was reported to accumulate in chloroplasts and then in nucleus, cytoplasm, peroxisomes (*82*) or mitochondoria (*83*, *84*), while superoxide ion formation has been reported in chloroplasts during photosynthesis (*85*) , in mitochondrial complexes (*85*), and in peroxisomes (*86*, *87*).

An example of where can $ONOO^-$ be generated in plant cells are chloroplasts. The chloroplasts are among the more active organelles involved in free energy transduction in plants (photo-phosphorylation). It has been reported that photosynthesis can be affected not only by generation of ROS (*88*), but also by reaction with NO and other NO-related species (*89*). Thus, the chloroplasts represent a place where $ONOO^-$ can form because of the simultaneous production of nitric oxide and photo-produced superoxide anion. Another cellular compartment with the capacity to generate and release both $O_2^{\bullet-}$ and NO into the cytosol are peroxisomes (*86*).

Peroxynitrite Implications in Plant Physiology and Hypersensitive Defense Response

There are interesting accounts of PON being present in living plants (*89*). However, thus far, the impact of this presence in plant physiology is insufficiently documented. Akin to the case of animal cells, peroxynitrite's involvement in plant cells and potential phytopathologic functions is not determined with certainty, partly due to its short half-life and high reactivity, which, to date, make it difficult to be studied directly with the available analytical tools (*vide infra*) (*72*).

For green plants, the nitric oxide-mediated signaling pathway in the defense against pathogens is widely accepted. In plants, NO might activate an iron regulatory protein (IRP) to increase in free iron concentration thereby promoting the Fenton reaction, which leads to formation of the hydroxyl radical $HO^\bullet$, thus creating a killing environment for both host and pathogen. Alternatively, $NO^\bullet$ reacts with $O_2^{\bullet-}$ to yield PON ($ONOO^-$), which was suggested to play an important role in the activation of the cell death program (*90*). An important distinction from the case of animal cells, is that $ONOO^-$ is not as destructive for plant cell metabolism as it is to animal systems. In fact, experimental data shows that exposure of soybean cells to exogenous PON did not cause cell death

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

at concentrations up to 1 mM, despite a strong increase in the pool of nitrated proteins. In contrast, for animal cells, concentrations as low as the micromolar range trigger a dose-dependent cell death.

In plant cells, nitrated proteins also serve as a marker of peroxinitrite accumulation. In this regard, several nitrated proteins have been reported in the context of the interaction of nitric oxide and reactive oxygen species in plant hypersensitive disease resistance response (*91*).

The significance of the post-translational protein nitration in the context of potential regulatory roles of PON in plant disease is still to be clearly established. Progress in this regard is limited by appropriate methods capable of monitoring dynamic concentrations of peroxynitrite.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

2.4. Is All Peroxynitrite Formed Accounted For? Analytical Challenges in the Face of Biological Complexity

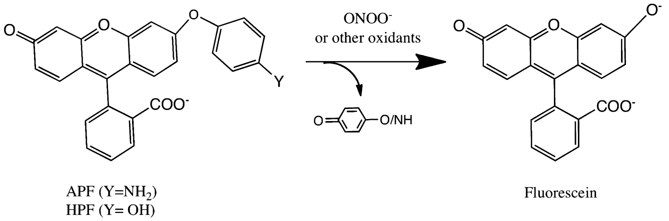
The preceding sections show but few highlights of the complexity of the biological chemistry of PON. The goal was not to provide an exhaustive review of peroxynitrite's biological mechanisms of action or implications in pathologies. Excellent reviews exist, which give very detailed accounts and in-depth description of peroxynitrite's biochemistry and pathophysiology (*12*, *13*, *18*, *72*, *92*). Our very brief overview, however, shows that the complex chemistry of this reactive nitrogen species and, as we will discuss later, the lack of versatile specific methods to follow it in biological systems, mask much of its mechanisms of actions.

Is all PON formed in biological systems accounted for? The question is valid even in cases of controlled experimental conditions *in vitro* such as the case of cultured cells. The answer is No. One obvious reason is that most analytical methods adopted use indirect assays targeting a particular biomarker, which cannot account for all peroxynitrite accumulation (*12*, *18*, *21*, *23*, *25*, *72*). In fact, attempts to quantify peroxynitrite-induced oxidative stress relied mainly on indirect detection of reaction products such as nitrated lipids and nitrated proteins using techniques such as chromatography, immunological techniques, and mass spectrometry. In this regard, detection of protein tyrosine nitration (3-nitrotyrosine) gained considerable importance in most investigations of PON-mediated cytotoxicity (*12*, *18*, *46*). However, the level of protein tyrosine nitration (and nitration of other aromatic aminoacids, e.g. tryptophanes) is very low and often does not provide consistent results with the extent of exposure to stress. The situation is even more complicated for *in vivo* systems; studies report very low yields of nitrotyrosine recovered under conditions of high peroxynitrite production, compared to experiments *in vitro* with direct exposure to exogenous peroxinitrite. In fact, under inflammatory conditions, nitrated tyrosines in plasma proteins represent at most 10 per 100,000 residues (*93*). Thus, tyrosine nitration seems to be a selective process that targets select proteins, and within each protein, specific tyrosine residues.

Moreover, tyrosine nitration is not strictly specific to PON (*94*), since the same modification can be caused by other mechanisms, such as the Fenton type iron/heme pathway (*95*), or through a reaction catalyzed by myeloperoxidase

(*96*), or other processes. Interestingly, and to this point, a mass spectrometric method that was developed to measure nitrotyrosine levels in smokers also found significant levels of the metabolite in the urine of nonsmokers. The problem of specificity is understandably complicated by the known multiple parallel reaction pathways (*97*), and a variety of target biomolecules encountered in vivo (*12*, *18*, *46*). Together, these observations establish that nitrotyrosine is not a specific indicator of PON formation, but rather a marker for a collection of pathways involving reactive nitrogen species. Given the multifaceted reactivity of peroxynitrite, one would need more than one method in order to account of this reactive analyte in a given biological system.

Other commonly used assays to determine peroxynitrite, particularly in cell-based studies, are based on fluorescence. Several fluorescence probes to detect peroxynitrite and other oxygen reactive species have been developed (*98–101*). For instance, the oxidation of non- or weakly-fluorescent compounds such as 2,7-dichlorodihydrofluorescein and dihydrorhodamine-123 by oxidants (including peroxynitrite) generate a dose-dependent fluorescence. However, these probes do not discriminate between oxidizing species such as hydrogen peroxide, nitric oxide, superoxide ion, or peroxynitrite (*98*, *100*).



Scheme 1. Reaction of aminophenylfluorescein (APF) or hydroxyphenylfluorescein (HPF) with strong oxidants such as peroxynitrite to generate fluorescein whose fluorescence is used to monitor the concentration of the oxidant.

In contrast, aminophenylfluorescein (APF) and hydroxyphenylfluorescein (HPF) that were developed later are particularly selective for the highly oxidant species (hydroxyl radicals, hypochlorite ion, and PON) but not for hydrogen peroxide or superoxide ion. In this case, strong oxidants such as peroxynitrite are able to drive the *O*-dearylation reaction that generates fluorescein, Scheme 1.

Given their differential fluorescence output for the various oxidants, APF and HPF can be used side by side to pinpoint a particular oxidant species (*100*). Although it is suggested that the kit can in principle distinguish between various species (e.g. NO• and HO• or ONOO− and NO•), the case cannot be made for hydroxyl radicals versus PON since their relative responses are not very distinct. Local environment and pH can generate significant amounts of hydroxyl radicals even if peroxynitrite is the primary product of oxidative stress. Also, the balance of NO to superoxide ion generated under stress, along with the right pH, may

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

provide conditions where another nitrogen oxide (dinitrogen trioxide) intermediate is formed, which would accelerate the process of formation of nitrogen dioxide radicals at the expense of peroxynitrite decay (*102*). In all of these cases specificity is critical in order to be able to link a biological observation to the molecule or molecules involved and the underlying mechanism of nitrative stress.

To sum up this section, our understanding of the mechanisms of action PON in biological systems is likely still at its infancy. Many roles of PON that we now know may well change, and others that we don't know are likely to see the light of day with better scrutiny of transient concentrations of this important biological molecule. Better and versatile analytical tools will enable that scrutiny.

In the next section, we provide a brief overview of the methods developed in this regard, recent accomplishments, the challenges faced, and opportunities of the near future.

3. Analytical Methods and Techniques for Detecting Peroxynitrite

Two recent reviews offered an analytical electrochemistry perspective (*103*) or a clinical-pharmacological perspective (*104*) on detecting PON. More specifically, the first review (*103*) discussed the main accomplishments and challenges faced in biological media (*vide supra*). The effect of potential interferents is particularly important when developing such electrochemical sensors. A brief outlook into the future refers to the use of multi-electrochemical array sensors for simultaneous detection of nitric oxide, superoxide and peroxynitrite.

The second review focused on measuring the reactive oxygen species (ROS; superoxide, hydrogen peroxide) and reactive nitrogen species (RNS; nitric oxide, peroxynitrite) within the vasculature (*104*). This is inherently difficult, due to their transient nature, low submicromolar concentrations within the vascular system, and their multifaceted reactivities. These key issues may influence the usefulness of a particular assay, when reemployed in vascular tissues matrices.

Herein, the current methods to detect PON are briefly discussed. We outline the most important contributions from the last ten years, classified as *non-electrochemical* or *electrochemical*, including recent ones coming from the authors of this chapter.

3.1. Non-Electrochemical Techniques for Peroxynitrite Detection

Non-electrochemical methods reported for PON detection comprise mainly optical techniques, typically luminescence and fluorescence. The fluorometric methods already discussed in the previous section are not repeated here.

Reports on fluorogenic sensors based on nanoparticles are increasingly on the rise. An example is a sensor that consists of nanoparticles coated with quenched oxazine fluorophores for monitoring peroxynitrite (ONOO-) and myeloperoxidase (MPO)-mediated hypochlorous acid/hypochlorite (HOCl/OCl-) production. The oxazine fluorophores were released specifically upon reaction with HOCl or

ONOO⁻. The platform is reported to be stable toward other oxidants such as hydroxyl radical, hydrogen peroxide, and superoxide (*105*). Recently, Yang et al. reported on their second-generation boron-dipyrromethene-based fluorescent probe (BODIPY) attached to another aromatic unit bearing a ketone group (4-hydroxyphenyl trifluorobutanone); upon reaction with PON, the ketone unit is transformed to a furan, resulting in fluorescence of the BODIPY component. The probe is reported to be highly sensitive and selective for peroxynitrite both as an added exogenous analyte and when generated *in situ* (e.g. in stimulated macrophage cells) (*106*).

Other reports used species endogenous to the biological medium. For instance, one method was reported on the ratiometric determination of PON using the aminoacid L-tyrosine and synchronous fluorescence spectroscopy (*107*). As an intrinsic fluorescent aminoacid, L-tyrosine reacts with ONO_2^- and CO_2, yielding a fluorescent tyrosine dimer in pH 8.5 buffer. The ratio of the synchronous emission bands, between the dimer (F-406) and of monomer (F-364), was quantitatively related to PON concentration. It was not clear if this method is affected by changes in experimental parameters. The limit of detection was 1.84×10^{-8} mol L^{-1} ONO_2^- and the linear range from 1.60×10^{-7} mol L^{-1} to 6×10^{-6} mol L^{-1} (*107*). A related method using hemoglobin as the catalyst and L-tyrosine as the substrate was also described (*108*). Folic acid is another biologically relevant molecule that was employed as a fluorescent probe: PON oxidizes the reduced, less fluorescent folic acid, to yield a higher fluorescent product. The increase in fluorescence was correlated to the concentration of PON (*101*). However, the applicability of this method to biological settings that intrinsically involve or can be affected by folic acid will not be straightforward.

Approaches based on separation methods such us capillary electrophoresis coupled to spectroscopic detection (*109*), and methods using perm-selective dialysis membranes with flow injection analysis based on chemiluminescence (*110*) were also reported. However, because of their nature, their potential for real-time monitoring and application to biological settings such as live cells is very limited.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

3.2. Electrochemical Methods for Peroxynitrite Detection

Direct electrochemical sensing is by far the method of choice that will fulfill the requirements of label-free and real-time monitoring, often desired for biological systems.

Although the interest in PON grew significantly since its biological relevance was first reported in early 1990s (*1*, *2*), data on its direct electrochemistry was lacking until a seminal report appeared in 2001 (*111*). In a thorough electrochemical investigation, Amatore et al. were able to characterize the direct electrochemical oxidation of PON at both platinized carbon fiber microelectrodes and platinum disc microelectrodes (*111*). A formal potential for the ONOO−/ONOO• redox couple was determined to be $E^o = 0.27$ V versus the SSCE reference electrode (*111*). Results from fast scan and steady-state voltammetry of PON, were used in the deconvolution of the current trace generated by oxidative bursts emitted by mechanically stimulated human

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

fibroblasts. Using this method, amperometric responses of ONOO−, and that of NO_2^-, H_2O_2, and NO• could be directly and simultaneously measured (*111*).

The separation of PON from other electroactive species and its amperometric detection was reported recently using microchip electrophoresis (MCE). The MCE allowed shorter (i.e. 25 s) separation times (*112*). The relatively higher temporal resolution compared to conventional capillary electrophoresis would permit a better tracking of dynamic chemical changes (*112*). The method showed evidence of short separation times, which can be beneficial particularly for fast decaying analytes such as PON. Although the method is off-line as described, it is conceivable to adapt it to cases where real-time monitoring is desired, such as near live cells.

Along the same lines, a concurrent amperometric detection of nitric oxide (NO) and peroxynitrite (ONOO-) was reported using an electrochemical sensor array chip (ESA), which has individually addressable sets of gold (Au) microelectrodes. The NO-sensitive microelectrodes are electrochemically modified by electrodepositing thin layers of poly(eugenol) and poly(phenol) to ensure selectivity. The amperometric detection was performed at +0.8 V vs. Ag/AgCl, as is the case for most NO sensors. In the same chamber, the microelectrodes for ONOO- determination are used without any chemical modification and operated at -0.1 V vs. Ag/AgCl. The potential and the selectivity against other intereferents were established in prior work by the same group (*113*). This less positive potential not only allows the reduction of peroxynitrous acid ONOOH in neutral pH (*113*), but also avoids interference of anions relevant to biological media.

This work successfully showed the proof of concept for simultaneous NO/ONOO- detection of the sensor array without crosstalk. It remains to be seen if this method can be adapted to measure NO and PON from cultured cells. In this regard, because of its intrinsic stability and diffusibility, NO monitoring from cultured cells in this device is not expected to face serious hurdles. This may not be the case for peroxynitrite, whose concentration profiles as released from cells may prove to be difficult to account for. Cell density, which sets average diffusion distances, may affect the "collection efficiencies" by the sensing electrode network and/or introduce variability from one individual electrode to the next in the same array. Variability of other environmental factors in the device, such as extracellular CO_2, may enhance the decay of the released PON and show apparent lower levels. Finally, an important problem that the choice of reduction (rather than oxidation at more positive potentials) may bring is the possibility to step on the onset of reduction of oxygen, which will be necessarily present in the cells medium. Even minor stepping on the electrochemical reduction wave of oxygen will potentially generate exogenous reduced oxygen species (superoxide and hydrogen peroxide). This "added external stress" may not only affect the balance of NO and PON after their release to the extracellular space, but also may bias the very intrinsic response of the cells. However, the platform as developed is valuable; it can for instance adopt oxidative potentials for the peroxynitrite-sensing electrode network but with adequate protective layers against interferents. A case in point for electrochemical detection based on oxidation of PON was reported (*114*). The authors used a conducting polymer-manganese ion complex, more specifically the (poly-di-thienyl-pyrrole-benzoicacid), termed as “Mn-pDPB” complex. The conducting polymer matrix is the result of electropolymerization of the DPB monomer. The polymer backbone provides binding sites for Mn^{2+} ions. A protective layer of polyethyleneimine (PEI) film ensures stability of the sensing surface and selectivity against major biological interferents. The PON amperometric calibration showed a dynamic range from 2×10^{-8} to 3×10^{-5} M, with a detection limit of 1.9×10^{-9} M.

Others showed that poly(cyanocobalamin) modified glassy carbon electrode prepared by electropolymerization could be used for voltammetric detection of ONOO-. The modified electrode seemed to exhibit an electrocatalytic activity for the ONOO- oxidation. Differential pulse voltammetry showed that the interface can be used to determine PON. The linear range was between 2×10^{-6} to 3.0×10^{-4} M with a detection limit of 10^{-7} M (*115*).

Although very limited in term of potential analytical applications in biological systems, Compton et al. described a method based on voltammetric detection of PON at a mercury film electrode (MFE) (*116*). The study showed the involvement of an ECE where the two electrochemical steps are in opposite direction which explains the unusual inverted peak observed in the presence of PON. The proposed mechanism suggests the initial formation of a thin Hg(I) oxide film on the electrode, which is then oxidized by ONOO- to form HgO in a chemical step. Then the HgO is reduced back to Hg(0) at the electrode potential of interest, resulting in a net cathodic current (*116*). However, as we stated earlier, this method is very limited in terms of real biological applications, since it suffers from a number of interferents including dioxygen (*116*).

Efforts to develop sensitive interfaces on electrodes for PON determination already showed that peroxynitrite interacts with a variety of transition metals, which could serve as electrocatalytic reporting sites. In this regard, a recent method from the authors’ labs, which will be detailed later in a separate section (*vide Infra*), uses the sensing performance of a polyethylenedioxythiophene (PEDOT)/hemin nano-structured film on carbon electrodes and carbon fiber microelectrodes. We showed that the modified interface could be used for electrocatalytic oxidation of PON. The sensitivity of the hemin-PEDOT modified CFEs was 13×10^{-9} A/10^{-6} M (*117*).

Details on the peroxynitrite detection work of the authors of this chapter follow in the next section (3.3)

3.3. Nanostructured Microsensors for Peroxynitrite Electrochemical Detection: Case of PEDOT/Hemin Nanostructured Catalytic Film

Methods for PON detection reported in the last decade or so include fluorescent probes, chemiluminescence, and electrochemical techniques. Although the consensus is that electrochemical detection tends to be faster, simpler, and amenable to application in biological systems, most recently published detection methods for peroxynitrite are based on fluorescence (*105–107*, *118–120*).

Electrochemical microsensors are capable to monitor the transient concentration gradients of redox-active species such as PON. Significantly, the

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

possibility to work with (sub)micron-sized tips provides fast response times and high spatial resolution, along with excellent analyte sensitivity. An elegant example in this regard is the simultaneous monitoring of peroxynitrite and other reactive species at an ultramicroelectrode placed 5 microns away from single human fibroblasts subjected to mechanical stimulation (*111*). Amperometric microsensors can easily be used in unstirred microenvironents, and thus seem ideal to measure local concentrations in semisolid samples with micron range spatial resolution (*121*, *122*).

In a series of investigation aiming at finding a suitable electrocatalyst for PON, we established that hemin can catalyze PON's oxidation (*117*). On the other hand, advances using electroactive polymers, also referred to as inherently conductive (e.g. polypyrrole or polythiophene), showed the potential of these materials for sensing and signal transduction. These "synthetic metals" exhibit unique properties, including mediating rapid electron transfer and direct communication, added to their ability to be synthesized under mild conditions, and deposited onto conductive surfaces from monomer solutions, with precise electrochemical control of their formation rate and thickness (*117*, *123*, *124*). In our effort to improve the sensitivity of our bare hemin electrodes, we investigated the quantification of PON using carbon-fiber microelectrodes modified with poly(3,4-ethylenedioxythiophene) or PEDOT and functionalized with hemin. The choice of the conducting polymer PEDOT was in part due to previous work that showed its biocompatibility. For instance, it was polymerized directly within living neural tissue to provide an electrically conductive network that is integrated within the tissue (*125*, *126*). Also, based on our experience, PEDOT with its tortuous surface with nanoscale features (*vide infra*) readily provides nanopaterned surface for catalyst loading, which results in an efficient nanostructured catalytic film.

In the following section we review the method of hemin-PEDOT film electropolymerization used for microsensor fabrication; we will describe the details of the preparation process, surface characterization of the PEDOT/hemin interface, and its performance in terms of PON detection.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

The Peroxynitrite Synthesis

Many methods exist for preparing peroxynitrite. We used the two-phase displacement reaction with the hydroperoxide anion in the aqueous phase and the isoamyl nitrite in the organic phase (*127*). PON remained in the aqueous phase, whereas the isoamyl alcohol formed a new organic phase along with the unreacted isoamyl nitrite. The aqueous phase contained some isoamyl alcohol and the unreacted hydrogen peroxide, but no isoamyl nitrite. Removal of isoamyl alcohol, or of any traces of isoamyl nitrite, was accomplished by washing the aqueous phase with dichloromethane and chloroform. The hydrogen peroxide is then removed by passing the solutions through a manganese dioxide column. PON, with a final concentration of up to 850 mM, was stored in 5 mL aliquots at minus-80°C, until ready to use.

Using the widely used molecular absorbtivity ε_{302} = 1705 mol^{-1} cm^{-1}, the concentration of PON as it forms in the reaction vessel can be followed by monitoring absorption at 302 nm (Figure 2).

For all subsequent tests and investigations, aliquots of frozen PON are thawed and kept on ice to minimize the spontaneous decay.

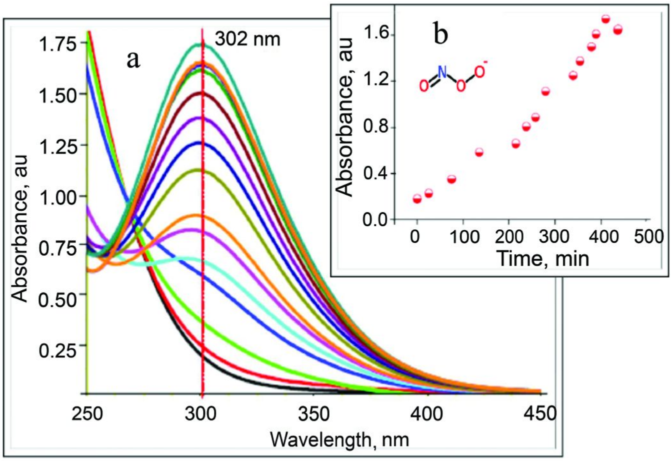


*Figure 2. The increase in relative absorbance indicates the formation of peroxynitrite (**a**), with its 302 nm peak intensity growing as a function of time (**b**). (see color insert)*

Electrochemistry

Electrochemical measurements were performed in standard two- or three-electrode cell configurations at room temperature 24 ± 2 °C. Other standard electrochemical details are as described in reference (*117*).

Preparation of the Carbon Fiber Electrodes (CFEs)

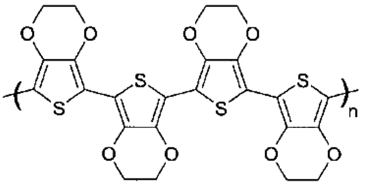
We prepared the carbon fiber microelectrodes according to published methods, described in detail elsewhere (*128*). The films (whether just hemin or the hemin-PEDOT configuration) were deposited on freshly cleaned 30-µm diameter, glass-encased CFEs. Prior to coating, the CFEs were sonicated in ethanol-acetone for 3 min. and then gently dried in a stream of nitrogen gas. After the electrochemical modification process, CFEs were gently rinsed with organic and aqueous solvents, to remove excess material, and then allowed to dry before use.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Preparation of the PEDOT-Functionalized Hemin

We used hemin (iron protoporphyrin IX), protoporphyrin and 3,4-ethylenedioxythiophene (EDOT) purchased from Sigma-Aldrich (St Louis, MO.). EDOT monomers are electroactive and upon electrooxidation they yield the corresponding PEDOT polymer, whose backbone is shown in Scheme 2.



Scheme 2. Backbone of poly(3,4-ethylenedioxythiophene) or PEDOT .

For hemin-only electrodes, the films were electropolymerized on freshly cleaned carbon fiber electrodes from a solution of 1.5 mM hemin monomer in dichloromethane in the presence of 0.1 M tetrabutylammonium tetrafluoroborate. The hemin-PEDOT film was electrodeposited from a solution of 1.5 mM hemin and 4.5 mM EDOT monomers in dichloromethane with 0.1 M tetrabutylammonium tetrafluoroborate as a supporting electrolyte. For hemin-PEDOT deposition, redox peak currents increased during repeated scanning for 10 cycles between –1.6 V and +1.6 V (Figure not shown) (*117*). The current increase is consistent with the formation of an electroactive multi-layered matrix deposited on the electrode’s surface, and that is capable of mediating the electrolysis of incoming porphyrin/EDOTsmonomer at the film-solution interface.

In the case of the electrodepositon of the hemin-only film, the surface coverage increased gradually through the first 10-15 scans and then hemin growth almost stops. In contrast, the PEDOT-Hemin multi-layered matrix exhibited an uninterrupted and continuous growth as judged by the uniform increase of peak currents for at least the first 40 scans. The inherent, relative high conductivity of the polythiophene polymeric film is responsible for this continuous film growth of the PEDOT-hemin layer. This difference between the two films is also apparent from their surface morphology, surface characterization profiles, and electrochemical response as will be shown later.

Surface Characterization

The surface morphology of the electrodeposited hemin films with and without PEDOT, compared with the bare carbon fiber, are shown in Figure 3.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

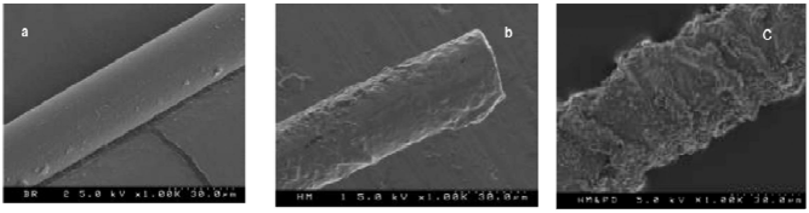


Figure 3. FESEM images of surface morphologies for (a) bare carbon fiber, (b) hemin-modified carbon fiber, and Hemin-PEDOT modified carbon fiber.

The bare CFE appeared flat, in contrast with the hemin electropolymerized film, which showed the growth of a film with a smooth surface and an apparent thickness of about 2 μm. By comparison, the hemin-PEDOT polymerized layer was much thicker, typically measuring about 8 μm and featuring a fractal and rough surface with peaks and valleys—at microscale

X-ray energy dispersive spectroscopy (XEDS) was used as a sensitive tool to analyze the composition of the catalytic layers. The XEDS characterization of hemin-PEDOT and protoporphyrin-PEDOT both show a typical sulfur peak (S = 16-19.7% wt), which is expected from the thiophene units in the PEDOT (Figure 4a).

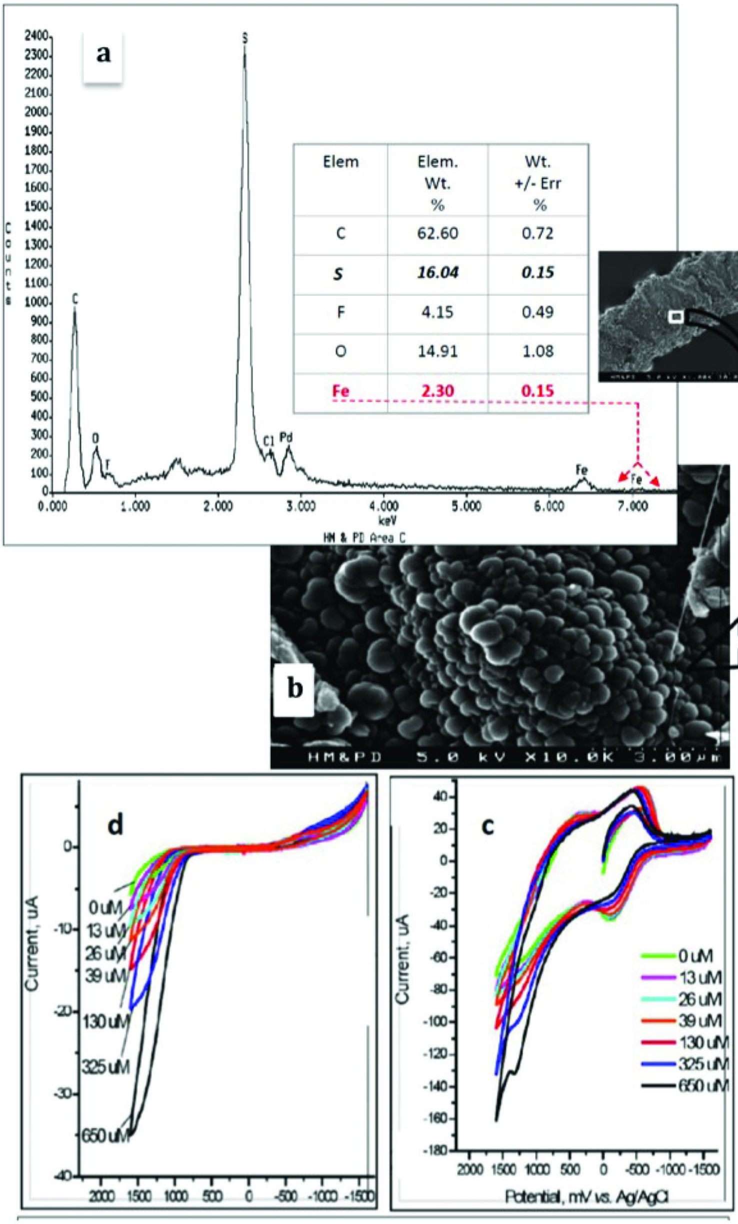
The iron peak is present in hemin-PEDOT (Fe = 2.3% wt), but absent for our control (Fe = 0.0% wt) with PEDOT-protoporphyrin (i.e. hemin macrocycle devoid of the central iron atom).

The high Magnification imaging (10,000x) reveals the nano-structured aspect of the surface, with features arranged in a 'cauliflower' type arrangement (Figure 4b). Dimensions of the nanostructured features are in the 100-300 nm range.

Cyclic Voltammetry

Cyclic voltammetry is a tool of choice to assess the catalytic activity and performance of the modified surface. It is also needed to select the right potential for time-based amperometric measurements. Figure 4c shows cyclic voltammograms of Hemin-PEDOT in 10.5 pH CAPS buffer solution in absence and presence of increasing concentration of PON. The cyclic voltammograms of the Hemin-PEDOT electrodes indicate two main electrode processes; first, a main oxidation peak, assigned to PEDOT, is close to 0 mV vs. Ag/AgCl, Notice that this feature is absent from the hemin-only electrode (Figure 4d). The peak around 0 mV vs. Ag/AgCl gives the possibility to detect PON with the electrode at 0 (zero) polarization in time-based amperometry. The possibility to work at low potentials automatically eliminates the possible interference from competing redox active substrates (particularly in biological systems) that typically oxidize at more positive potentials (100-700 mV range). The second oxidation peak at ~ +1250 mV is characteristic of hemin.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011



Elem	Elem. Wt. %	Wt. +/- Err %
C	62.60	0.72
S	***16.04***	***0.15***
F	4.15	0.49
O	14.91	1.08
Fe	**2.30**	**0.15**

*Figure 4. (**a**) XEDS analysis shows the typical sulfur and iron peaks expected in the thiophene/iron porphyrin units in the film. (**b**) FESEM images showing the nano-structured Hemin-PEDOT film (**c**) Typical voltammetric responses of the PEDOT/Hemin modified electrodes to peroxynitrite additions. (**d**) Response of hemin-only film. (see color insert)*

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Hemin/PEDOT as an Amperometric Microsensor

Cyclic voltammetry showed that the oxidation currents recorded on the modified electrode increased with the $ONOO^-$ concentration. We used this characteristic in time-based amperometry to evaluate the performance of Hemin-PEDOT-modified microelectrode as a PON-sensitive microsensor. For these tests, the working electrode was polarized at +750 mV versus Ag/AgCl. Amperometric currents were measured in response to varying amounts of peroxynitrite added to CAPS buffer solution. A typical dose-response current trace is shown in Figure 5a. Arrows indicate the times of addition of $ONOO^-$ aliquots to the buffer solution. The figure shows that the amperometric response of Hemin-PEDOT microsensor is stable and reproducible for repeated measurements. The response of this microsensor is compared with the PEDOT–only, Hemin-only, and bare CFE.

We studied the basic characteristics defining the performance of the sensor including the detection limit, the response time, concentration range, the sensitivity and selectivity, the pH dependence and the reproducibility, repeatability and storage stability. The reader is referred to the detailed study published recently (*117*). Briefly, these hemin-PEDOT modified CFEs showed a sensitivity of 13 nA/μM, i.e. 52 times higher than the hemin-modified CFE and 130 times higher than the bare CFE.

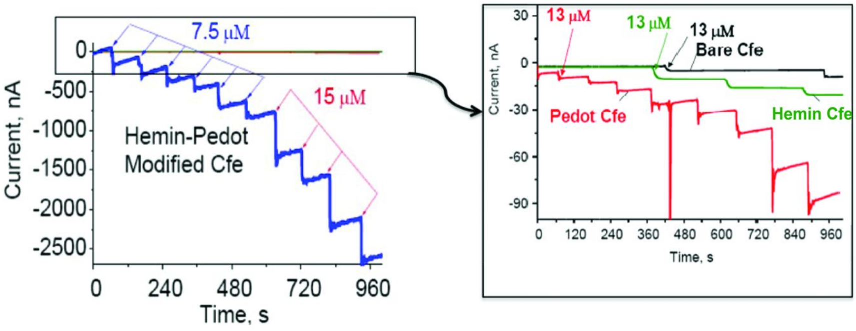


*Figure 5. The chronoamperometric responses of Hemin-PEDOT film on carbon microelectrode to added peroxynitrite additions (Left Panel). Compared responses of bare fiber (**black trace**), hemin only (**green trace**), PEDOT only (**red trace**), and hemin-PEDOT (Right Panel). (see color insert)*

The typical calibration curves for low and large concentration ranges from amperometric tests are shown in Figure 6a with a focus on for 0-to-5x10^{-6}M analyte concentration range. Figure 6b shows amperometric response of the Hemin-PEDOT sensor at low concentrations of PON. The detection limit that we obtained with these modified microelectrodes is ~200 nM peroxynitrite based on the 3-to-1 response-to-noise ratio rule. Typical response times for the modified carbon fiber electrodes were between 5 s and 30 s, depending on the fiber length, and the thickness of the catalyst film, which in turn depends on the duration of the electrodeposition.

We assessed the selectivity of the Hemin-PEDOT microsensor for PON against nitrite and nitrate. This is because the main decomposition products of peroxinitrite are nitrite and nitrate with a product distribution that depends on factors such as pH and temperature. We have therefore compared the response of our modified electrodes to peroxynitrite to the response observed for nitrite and nitrate. We show that the Hemin-PEDOT modified electrodes are inherently very selective and more responsive to additions of PON compared to nitrite or nitrate (*117*).

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

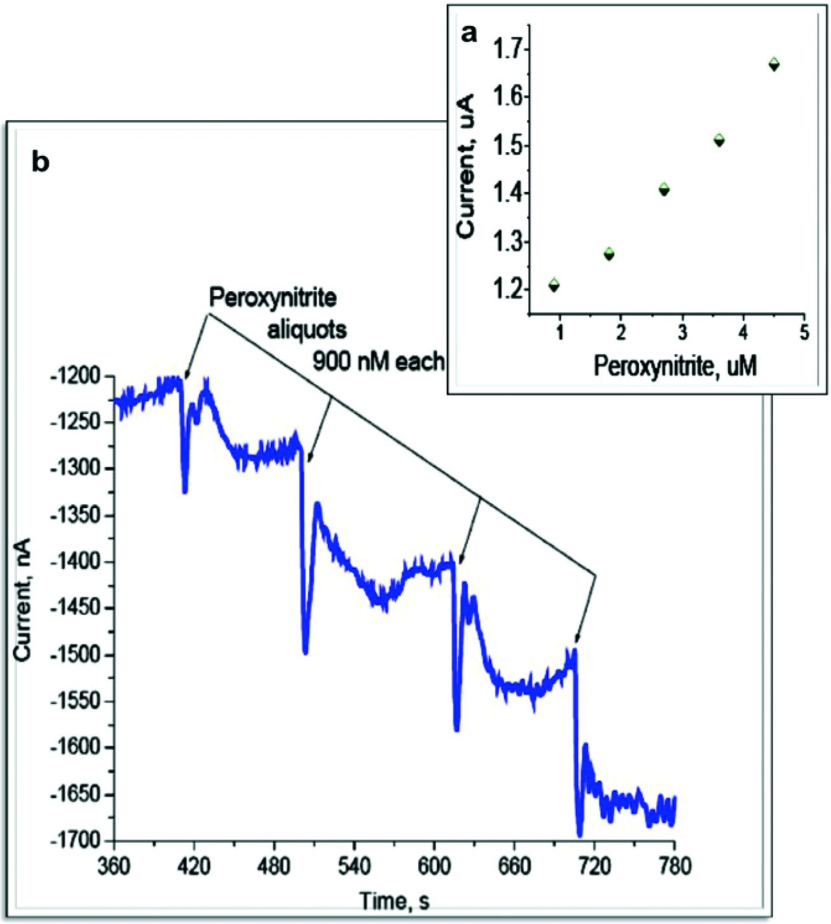


Figure 6. ***(a)*** *Calibration curve for Hemin-PEDOT amperometric response to peroxynitrite additions in the micromolar range.* ***(b)*** *Response of the same sensor to peroxynitrite concentrations in the high nanomolar range. (see color insert)*

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

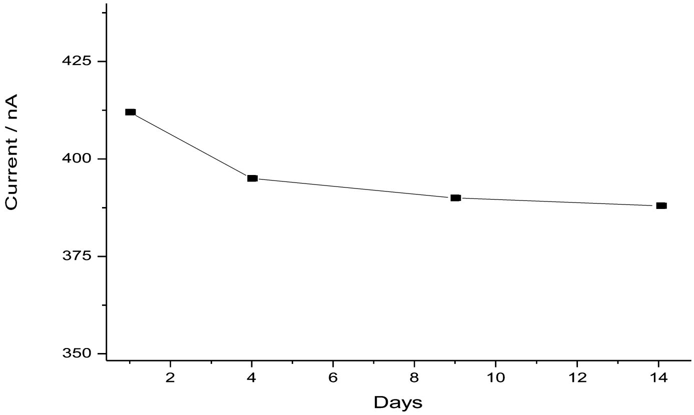


Figure 7. Response of a Hemin-PEDOT microsensor to peroxynitrite over two weeks with intermittent storage at in a sealed container at 4 °C.

In terms of response stability, the Hemin-PEDOT sensors generally showed very stable responses from observation of the microsensor's performance over two weeks under the same conditions. Between measurements, the microsensor was stored dry in a sealed tube at 4°C. Except for a small decrease in response for the first couple of days (~5%), the response was essentially stable over the remainder of the following two weeks (Figure 7).

In summary, the data presented in this section shows that the Hemin-PEDOT surface provides an efficient sensing platform for PON. While the catalytic oxidation of the analyte is mediated by the iron-protoporphyrin IX units, the PEDOT electroactive polymeric matrix brings synergy to the process: Not only it ensures efficient charge transport to support the catalytic oxidation, but it also allows high exposure of hemin catalytic sites through its inherent tortuous surface and nanostructured features as evidenced by FESEM imaging. This configuration maximizes the catalyst loading on the surface and enhances the access of PON to catalytic sites. As a result, the Hemin–PEDOT modified carbon microelectrodes exhibit low detection limit and high sensitivity.

Hemin as a catalyst in the PEDOT matrix provided a proof of concept of this "two-component" microsensor configuatrion. We have now explored other electrocalalysts, including some manganese porphyrins, on the PEDOT matrix in order to improve the sensor's sensitivity, detection limit, and selectivity.

4. Conclusions and Future Outlook

The true role and actions of PON in cellular biology are still blurred, if not masked, by its multifaceted chemical reactivity with many cell components. While the pathological impact of this reactive species just started to reveal the face of this "ugly biofactor" (*13*), other potentially beneficial roles (*5–10*) that may be intrinsic to regulatory "backdoors" gated by this species are relatively rarely mentioned or explored.

However, with just two *short* decades, the interest in the biological implications of this molecule still grew tremendously. In fact, if the number of citations of the word "peroxynitrite" in the Title of published research articles can serve as a metric, there was hardly any paper (~3) published before 1990 based on Thomson Reuters' Web of Science citation database. From 1990 to present (2011), however, the database shows around 11,260! (Figure 8A). This is an incredible jump indeed following the seminal report by Beckman and collaborators. If we use the same metric as above, the decade following the year 1990 brought ~3,970 articles bearing peroxynitrite in the title, whereas the period from 2000 to 2011, added ~7,290 articles on peroxynitrite; the number of the last decade amounts to ~180% jump over the 1990s, and represents ~65% of all articles since peroxynitrite was brought to the "biological spotlight" in early 1990s (Figure 8A). These numbers show that the interest and the growth in research output in the area of peroxynitrite's role in health and disease is decisively accelerating, Figure 8A.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

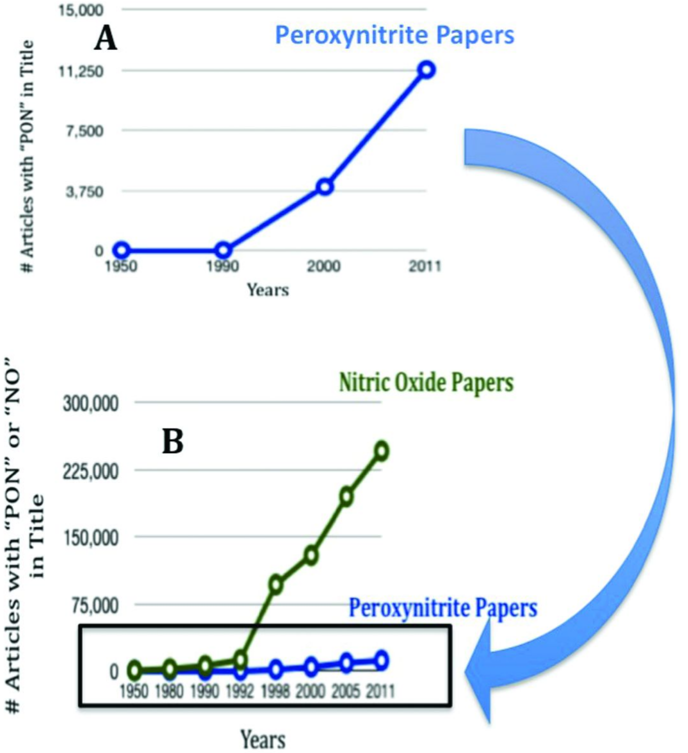


*Figure 8. **(A)** Number of research articles bearing "peroxynitrite, PON" in the Title as a function of years. Data from Thomson Reuters' Web of Science database. **(B)** Number of nitric oxide articles and peroxynitrite articles as a function of years. Data from Thomson Reuters' Web of Science database. (see color insert)*

However, the level of progress on the peroxynitrite research front is still relatively “mute” compared to that of the parent molecule and senior relative: nitric oxide. Figure 8B mputs things in perspective. It is still a comparison using the same metric: the number of research article having in the title “Nitric Oxide” compared to those bearing “Peroxynitrite”. One does not have to take much time to convey the simple fact: Research output with focus on peroxynitrite is still trailing at basal levels compared to nitric oxide.

Interestingly, this picture correlates with -or mirrors- our levels of understanding of the chemical and cellular biology of the two analytes. Of course, analytical chemistry with the tools and assays as applied to peroxynitrite’s chemistry in biological systems is an important determinant in unlocking this situation in the near future. The progress made so far on this front, mainly with optical methods and related probes, is commendable and did open interesting research directions pertaining to peroxynitrite’s role in living systems, or in exploring potential therapeutics.

There is still much to do for the development of more sensitive and more specific probes for optical methods. There is even more to do to develop better electrochemical sensors that are very sensitive and selective for PON in the biological medium or even *in vivo*. Better, electrochemical probes with these desirable characteristics and that can be miniaturized to fit closer to live cells, and report on their level of nitrative stress, have the potential to shed light on peroxynitrite’s biochemical dark side. Microchips and miniaturized electrochemical array sensors capable of monitoring simultaneously multiple analytes of nitroxidative stress are definitely needed. In fact, as we reviewed earlier in the section about the cellular chemistry of PON, not only the absolute amounts of the various oxygen and nitrogen reactive analytes is of value under a certain stress condition, but the balance or ratio of their concentrations is of more value, and can define various risk levels for nitroxidative stress.

Lastly, Figure 8B points to another interesting fact. The years following 1992 marked an enormous jump in research published on nitric oxide. Interestingly, 1992 was the year that Science Magazine named nitric oxide “Molecule Of The Year” (*129*). Figure 8B shows that the exponential growth continued even past 1998, the year where the Nobel Prize in Physiology or Medicine was awarded for discoveries of nitric oxide’s biological roles. An important arsenal that supported this growth was the collection of analytical tools used to monitor nitric oxide as well as its biological targets.

What the near future holds for peroxynitrite? “Molecule of the Year?” May be. While it is not officially named as such it certainly is already, given the critical mass of research on this molecule, and recent revelations about its important physiological and pathobiological roles. Our understanding in this regard is -and will be- as good as our analytical tools. It will depend on our ability to follow both pulsed and steady-state concentrations of this species generated under various stress –and may be normal?- conditions.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Acknowledgments

This research was supported by the National Science Foundation (MB; Grant CHE-0848820) and by an FRD and seed grants from CSU-Ohio Board of Regents. SFP acknowledges initial support from the CIES, through a Senior Fellowship in Biological Sciences from the J.William Fulbright Foundation.

References

1. Beckman, J. S. *Nature* **1990**, *345*, 27–28.
2. Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 1620–1624.
3. Khan, M. T.; Furchgott, R. F. *Federation Proceedings* **1987**, *46*, 385.
4. Schildknecht, S.; Bachschmid, M.; Ullrich, V. *FASEB J.* **2005**, *19*, 1169–1171.
5. Lefer, D. J.; Scalia, R.; Campbell, B.; Nossuli, T.; Hayward, R.; Salamon, M.; Grayson, J.; Lefer, A. M. *J. Clin. Invest.* **1997**, *99*, 684–691.
6. Nossuli, T. O.; Hayward, R.; Scalia, R.; Lefer, A. M. *Circulation* **1997**, *96*, 2317–2324.
7. Altug, S.; Demiryurek, A. T.; Kane, K. A.; Kanzik, I. *Br. J. Pharmacol.* **2000**, *130*, 125–131.
8. Hosgor-Basgut, B.; Basgut, E.; Soyak, G.; Tunctan, B.; Cakici, I.; Kanzik, I. *Life Sci.* **2000**, *67*, 3123–3127.
9. Altup, S.; Demiryurek, A. T.; Ak, D.; Tungel, M.; Kanzik, I. *Eur. J. Pharmacol.* **2001**, *415*, 239–246.
10. Bishop, A.; Gooch, R.; Eguchi, A.; Jeffrey, S.; Smallwood, L.; Anderson, J.; Estevez, A. G. *J. Neurochem.* **2009**, *109*, 74–84.
11. Blough, N. V.; Zafiriou, O. C. *Inorg. Chem. Res. Toxicol.* **1985**, *24*, 3502–3504.
12. Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev.* **2007**, *87*, 315–424.
13. Ascenzi, P.; di Masi, A.; Sciorati, C.; Clementi, E. *Biofactors* **2010**, *36*, 264–273.
14. Radi, R.; Peluffo, G.; Alvarez, M. N.; Naviliat, M.; Cayota, A. *Free Radical Biol. Med.* **2001**, *30*, 463–488.
15. Alvarez, M. N.; Piacenza, L.; Irigoin, F.; Peluffo, G.; Radi, R. *Arch. Biochem. Biophys.* **2004**, *432*, 222–232.
16. Denicola, A.; Souza, J. M.; Radi, R. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 3566–3571.
17. Marla, S. S.; Lee, J.; Groves, J. T. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 14243–14248.
18. Szabo, C.; Ischiropoulos, H.; Radi, R. *Nat. Rev. Drug Discovery* **2007**, *6*, 662–680.
19. Bartesaghi, S.; Valez, V.; Trujillo, M.; Peluffo, G.; Romero, N.; Zhang, H.; Kalyanaraman, B.; Radi, R. *Biochemistry* **2006**, *45*, 6813–6825.
20. Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. *J. Biol. Chem.* **1991**, *266*, 4244–4250.

21. Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Nitric Oxide* **2006**, *14*, 109–121.
22. Nalwaya, N.; Deen, W. M. *Chem. Res. Toxicol.* **2005**, *18*, 486–493.
23. Quijano, C.; Romero, N.; Radi, R. *Free Radical Biol. Med.* **2005**, *39*, 728–741.
24. Rubbo, H.; Radi, R.; Trujillo, M.; Telleri, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B. A. *J. Biol. Chem.* **1994**, *269*, 26066–26075.
25. Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. *Arch. Biochem. Biophys.* **1992**, *298*, 431–437.
26. MacMillan-Crow, L. A.; Crow, J. P.; Thompson, J. A. *Biochemistry* **1998**, *37*, 1613–1622.
27. Alvarez, B.; Demicheli, V.; Duran, R.; Trujillo, M.; Cervenansky, C.; Freeman, B. A.; Radi, R. *Free Radical Biol. Med.* **2004**, *37*, 813–822.
28. Savvides, S. N.; Scheiwein, M.; Bohme, C. C.; Arteel, G. E.; Karplus, P. A.; Becker, K.; Schirmer, R. H. *J. Biol. Chem.* **2002**, *277*, 2779–2784.
29. Szabo, C.; Ischiropoulos, H.; Radi, R. *Nat. Rev. Drug Discovery* **2007**, *6*, 662–680.
30. Van der Vliet, A.; Smith, D.; O'Neill, C. A.; Kaur, H.; Darley-Usmar, V.; Cross, C. E.; Halliwell, B. *Biochem. J.* **1994**, *303* (Part 1), 295–301.
31. Kuzkaya, N.; Weissmann, N.; Harrison, D. G.; Dikalov, S. *J. Biol. Chem.* **2003**, *278*, 22546–22554.
32. Zou, M.; Yesilkaya, A.; Ullrich, V. *Drug Metab. Rev.* **1999**, *31*, 343–349.
33. Grover, A. K.; Kwan, C. Y.; Samson, S. E. *Am. J. Physiol. Cell Physiol.* **2003**, *285*, C1537–C1543.
34. Blanchard-Fillion, B.; Souza, J. M.; Friel, T.; Jiang, G. C.; Vrana, K.; Sharov, V.; Barron, L.; Schoneich, C.; Quijano, C.; Alvarez, B.; Radi, R.; Przedborski, S.; Fernando, G. S.; Horwitz, J.; Ischiropoulos, H. *J Biol Chem* **2001**, *276*, 46017–46023.
35. van der Vliet, A.; Hristova, M.; Cross, C. E.; Eiserich, J. P.; Goldkorn, T. *J. Biol. Chem.* **1998**, *273*, 31860–31866.
36. Paxinou, E.; Chen, Q.; Weisse, M.; Giasson, B. I.; Norris, E. H.; Rueter, S. M.; Trojanowski, J. Q.; Lee, V. M.; Ischiropoulos, H. *J. Neurosci.* **2001**, *21*, 8053–8061.
37. Landino, L. M.; Skreslet, T. E.; Alston, J. A. *J. Biol. Chem.* **2004**, *279*, 35101–35105.
38. Park, S. U.; Ferrer, J. V.; Javitch, J. A.; Kuhn, D. M. *J. Neurosci.* **2002**, *22*, 4399–4405.
39. Dairou, J.; Atmane, N.; Rodrigues-Lima, F.; Dupret, J. M. *J. Biol. Chem.* **2004**, *279*, 7708–7714.
40. Dairou, J.; Dupret, J. M.; Rodrigues-Lima, F. *FEBS Lett.* **2005**, *579*, 4719–4723.
41. Mohr, S.; Stamler, J. S.; Brune, B. *FEBS Lett.* **1994**, *348*, 223–227.
42. Kennedy, L. J.; Moore, K., Jr.; Caulfield, J. L.; Tannenbaum, S. R.; Dedon, P. C. *Chem. Res. Toxicol.* **1997**, *10*, 386–392.
43. Pacher, P.; Szabo, C. *Am. J. Pathol.* **2008**, *173*, 2–13.
44. Beller, C. J.; Radovits, T.; Kosse, J.; Gero, D.; Szabo, C.; Szabo, G. *J. Vasc. Surg.* **2006**, *43*, 824–830.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

45. Scott, G. S.; Szabo, C.; Hooper, D. C. *J. Neurotrauma* **2004**, *21*, 1255–1263.
46. Szabo, C. *Toxicol. Lett.* **2003**, *140-141*, 105–112.
47. Lee, J.; Hunt, J. A.; Groves, J. T. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2913–2918.
48. Santos, C. X.; Anjos, E. I.; Augusto, O. *Arch. Biochem. Biophys.* **1999**, *372*, 285–294.
49. Kuzkaya, N.; Weissmann, N.; Harrison, D. G.; Dikalov, S. *Biochem. Pharmacol.* **2005**, *70*, 343–354.
50. Bryk, R.; Griffin, P.; Nathan, C. *Nature* **2000**, *407*, 211–215.
51. Piacenza, L.; Alvarez, M. N.; Peluffo, G.; Radi, R. *Curr. Opin. Microbiol.* **2009**, *12*, 415–421.
52. Stern, M. K.; Jensen, M. P.; Kramer, K. *J. Am. Chem. Soc.* **1996**, *118*, 8735–8736.
53. Hunt, J. A.; Lee, J.; Groves, J. T. *Chem. Biol.* **1997**, *4*, 845–58.
54. Shimanovich, R.; Groves, J. T. *Arch Biochem Biophys* **2001**, *387*, 307–317.
55. Shimanovich, R.; Hannah, S.; Lynch, V.; Gerasimchuk, N.; Mody, T. D.; Magda, D.; Sessler, J.; Groves, J. T. *J. Am. Chem. Soc.* **2001**, *123*, 3613–3614.
56. Sharma, S. S.; Munusamy, S.; Thiyagarajan, M.; Kaul, C. L. *J. Neurosurg.* **2004**, *101*, 669–675.
57. Thiyagarajan, M.; Kaul, C. L.; Sharma, S. S. *Br. J. Pharmacol.* **2004**, *142*, 899–911.
58. Lancel, S.; Tissier, S.; Mordon, S.; Marechal, X.; Depontieu, F.; Scherpereel, A.; Chopin, C.; Neviere, R. *J Am. Coll. Cardiol.* **2004**, *43*, 2348–2358.
59. Chirino, Y. I.; Hernandez-Pando, R.; Pedraza-Chaverri, J. *BMC Pharmacol.* **2004**, *4*, 20.
60. Mabley, J. G.; Liaudet, L.; Pacher, P.; Southan, G. J.; Groves, J. T.; Salzman, A. L.; Szabo, C. *Mol. Med.* **2002**, *8*, 581–590.
61. Jiao, X. Y.; Gao, E.; Yuan, Y.; Wang, Y.; Lau, W. B.; Koch, W.; Ma, X. L.; Tao, L. *J. Pharmacol. Exp. Ther.* **2009**, *328*, 777–784.
62. Bianchi, C.; Wakiyama, H.; Faro, R.; Khan, T.; McCully, J. D.; Levitsky, S.; Szabo, C.; Sellke, F. W. *Ann. Thorac. Surg.* **2002**, *74*, 1201–1207.
63. Nin, N.; El-Assar, M.; Sanchez, C.; Ferruelo, A.; Sanchez-Ferrer, A.; Martinez-Caro, L.; Rojas, Y.; Paula, M.; Hurtado, J.; Esteban, A.; Lorente, J. A. *Shock* **2011**, *36*, 156–161.
64. Bolwell, G. P. *Curr. Opin. Plant Biol.* **1999**, *2*, 287–294.
65. Durner, J.; Klessig, D. F. *Curr. Opin. Plant Biol.* **1999**, *2*, 369–374.
66. Alamillo, J. M.; Garcia-Olmedo, F. *Plant J.* **2001**, *25*, 529–540.
67. Garcia-Olmedo, F.; Rodriguez-Palenzuela, P.; Molina, A.; Alamillo, J. M.; Lopez-Solanilla, E.; Berrocal-Lobo, M.; Poza-Carrion, C. *FEBS Lett.* **2001**, *498*, 219–222.
68. Delledonne, M.; Zeier, J.; Marocco, A.; Lamb, C. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 13454–13459.
69. Perazzolli, M.; Romero-Puertas, M. C.; Delledonne, M. *J. Exp. Bot.* **2006**, *57*, 479–488.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

70. Tada, Y.; Mori, T.; Shinogi, T.; Yao, N.; Takahashi, S.; Betsuyaku, S.; Sakamoto, M.; Park, P.; Nakayashiki, H.; Tosa, Y.; Mayama, S. *Mol. Plant-Microbe Interact.* **2004**, *17*, 245–253.
71. Shimoda, Y.; Nagata, M.; Suzuki, A.; Abe, M.; Sato, S.; Kato, T.; Tabata, S.; Higashi, S.; Uchiumi, T. *Plant Cell Physiol.* **2005**, *46*, 99–107.
72. Arasimowicz-Jelonek, M.; Floryszak-Wieczorek, J. *Phytochemistry* **2011**, *72*, 681–688.
73. Saito, S.; Yamamoto-Katou, A.; Yoshioka, H.; Doke, N.; Kawakita, K. *Plant Cell Physiol.* **2006**, *47*, 689–697.
74. Torres, M. A.; Dangl, J. L.; Jones, J. D. G. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 517–522.
75. Yoshioka, H.; Asai, S.; Yoshioka, M.; Kobayashi, M. *Mol. Cells* **2009**, *28*, 321–329.
76. Guo, F. Q.; Okamoto, M.; Crawford, N. M. *Science* **2003**, *302*, 100–103.
77. Zeidler, D.; Zahringer, U.; Gerber, I.; Dubery, I.; Hartung, T.; Bors, W.; Hutzler, P.; Durner, J. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15811–15816.
78. Desikan, R.; Griffiths, R.; Hancock, J.; Neill, S. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 16314–16318.
79. Yamasaki, H.; Sakihama, Y. *FEBS Lett.* **2000**, *468*, 89–92.
80. Bethke, P. C.; Badger, M. R.; Jones, R. L. *Plant Cell* **2004**, *16*, 332–341.
81. Cooney, R. V.; Harwood, P. J.; Custer, L. J.; Franke, A. A. *Environ. Health Perspect.* **1994**, *102*, 460–462.
82. Foissner, I.; Wendehenne, D.; Langebartels, C.; Durner, J. *Plant J.* **2000**, *23*, 817–824.
83. Guo, F. Q.; Crawford, N. M. *Plant Cell* **2005**, *17*, 3436–3450.
84. Planchet, E.; Gupta, K. J.; Sonoda, M.; Kaiser, W. M. *Plant J.* **2005**, *41*, 732–743.
85. Gill, S. S.; Tuteja, N. *Plant Physiol. Biochem.* **2010**, *48*, 909–930.
86. Corpas, F. J.; Barroso, J. B.; del Rio, L. A. *Trends Plant Sci.* **2001**, *6*, 145–150.
87. del Rio, L. A.; Corpas, F. J.; Leon, A. M.; Barroso, J. B.; Carreras, A.; Sandalio, L. M.; Palma, J. M. *Free Radical Biol. Med.* **2004**, *36*, S41.
88. Asada, Y.; Miyake, M.; Miyake, J.; Kurane, R.; Tokiwa, Y. *Int. J. Biol. Macromol.* **1999**, *25*, 37–42.
89. Jasid, S.; Simontacchi, M.; Bartoli, C. G.; Puntarulo, S. *Plant Physiol.* **2006**, *142*, 1246–1255.
90. Wendehenne, D.; Pugin, A.; Klessig, D. F.; Durner, J. *Trends Plant Sci.* **2001**, *6*, 177–183.
91. Cecconi, D.; Orzetti, S.; Vandelle, E.; Rinalducci, S.; Zolla, L.; Delledonne, M. *Electrophoresis* **2009**, *30*, 2460–2468.
92. Salvemini, D.; Neumann, W. *Life Sci.* **2010**, *86*, 604–614.
93. Souza, J. M.; Peluffo, G.; Radi, R. *Free Radical Biol. Med.* **2008**, *45*, 357–366.
94. Halliwell, B. *FEBS Lett* **1997**, *411*, 157–160.
95. Thomas, D. D.; Espey, M. G.; Vitek, M. P.; Miranda, K. M.; Wink, D. A. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 12691–12696.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

96. Eiserich, J. P.; Hristova, M.; Cross, C. E.; Jones, A. D.; Freeman, B. A.; Halliwell, B.; van der Vliet, A. *Nature* **1998**, *391*, 393–397.
97. Ferrer-Sueta, G.; Radi, R. *Acs Chem. Biol.* **2009**, *4*, 161–177.
98. Hempel, S. L.; Buettner, G. R.; O'Malley, Y. Q.; Wessels, D. A.; Flaherty, D. M. *Free Radical Biol. Med.* **1999**, *27*, 146–159.
99. Yang, X. F.; Guo, X. Q.; Zhao, Y. B. *Talanta* **2002**, *57*, 883–890.
100. Setsukinai, K.; Urano, Y.; Kakinuma, K.; Majima, H. J.; Nagano, T. *J. Biol. Chem.* **2003**, *278*, 3170–3175.
101. Huang, J. C.; Li, D. J.; Diao, J. C.; Hou, J.; Yuan, J. L.; Zou, G. L. *Talanta* **2007**, *72*, 1283–1287.
102. Goldstein, S.; Czapski, G.; Lind, J.; Merenyi, G. *Chem. Res. Toxicol.* **1999**, *12*, 132–136.
103. Bedioui, F.; Quinton, D.; Griveau, S.; Nyokong, T. *Phys. Chem. Chem. Phys.* **2010**, *12*, 9976–9988.
104. Tarpey, M. M.; Fridovich, I. *Circ. Res.* **2001**, *89*, 224–236.
105. Panizzi, P.; Nahrendorf, M.; Wildgruber, M.; Waterman, P.; Figueiredo, J. L.; Aikawa, E.; McCarthy, J.; Weissleder, R.; Hilderbrand, S. A. *J. Am. Chem. Soc.* **2009**, *131*, 15739–15744.
106. Sun, Z. N.; Wang, H. L.; Liu, F. Q.; Chen, Y.; Tam, P. K. H.; Yang, D. *Org. Lett.* **2009**, *11*, 1887–1890.
107. Du, J. O.; Guo, X. Q. *Spectrosc. Spectral Anal.* **2008**, *28*, 1875–1878.
108. Liang, J.; Liu, Z. H.; Cai, R. X. *Anal. Chim. Acta* **2005**, *530*, 317–324.
109. Frankenfeld, C. N.; Rosenbaugh, M. R.; Fogarty, B. A.; Lunte, S. M. *J. Chromatogr., A* **2006**, *1111*, 147–152.
110. Dai, K.; Vlessidis, A. G.; Evmiridis, N. P. *Talanta* **2003**, *59*, 55–65.
111. Amatore, C.; Arbault, S.; Bruce, D.; de Oliveira, P.; Erard, M.; Vuillaume, M. *Chem.−Eur. J.* **2001**, *7*, 4171–4179.
112. Hulvey, M. K.; Frankenfeld, C. N.; Lunte, S. M. *Anal. Chem.* **2010**, *82*, 1608–1611.
113. Quinton, D.; Griveau, S.; Bedioui, F. *Electrochem. Commun.* **2010**, *12*, 1446–1449.
114. Koh, W. C. A.; Son, J. I.; Choe, E. S.; Shim, Y. B. *Anal. Chem.* **2010**, *82*, 10075–10082.
115. Wang, Y.; Chen, Z. Z. *Talanta* **2010**, *82*, 534–539.
116. Zakharova, E. A.; Yurmazova, T. A.; Nazarov, B. F.; Wildgoose, G. G.; Compton, R. G. *New J. Chem.* **2007**, *31*, 394–400.
117. Peteu, S.; Peiris, P.; Gebremichael, E.; Bayachou, M. *Biosens. Bioelectron.* **2010**, *25*, 1914–1921.
118. Cao, Q. H.; Zhou, Q. X.; Cai, R. X.; Liu, Z. H. *Anal. Sci.* **2005**, *21*, 445–447.
119. Martin-Romero, F. J.; Gutierrez-Martin, Y.; Henao, F.; Gutierrez-Merino, C. *J. Fluoresc.* **2004**, *14*, 17–23.
120. Yang, D.; Wang, H. L.; Sun, Z. N.; Chung, N. W.; Shen, J. G. *J. Am. Chem. Soc.* **2006**, *128*, 6004–6005.
121. Peteu, S. F.; Emerson, D.; Worden, R. M. *Biosens. Bioelectron.* **1996**, *11*, 1059–1071.
122. Peteu, S. F.; Widman, M. T.; Worden, R. M. *Biosens. Bioelectron.* **1998**, *13*, 1197–1203.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

123. Wallace, G. G.; Smyth, M.; Zhao, H. *TrAC, Trends Anal. Chem.* **1999**, *18*, 245–251.
124. Vidal, J. C.; Garcia-Ruiz, E.; Castillo, J. R. *Microchim. Acta* **2003**, *143*, 93–111.
125. Ludwig, K. A.; Uram, J. D.; Yang, J.; Martin, D. C.; Kipke, D. R. *J. Neural. Eng.* **2006**, *3*, 59–70.
126. Richardson-Burns, S. M.; Hendricks, J. L.; Martin, D. C. *J. Neural. Eng.* **2007**, *4*, L6–L13.
127. Uppu, R. M.; Pryor, W. A. *Anal. Biochem.* **1996**, *236*, 242–249.
128. Kawagoe, K. T.; Zimmerman, J. B.; Wightman, R. M. *J. Neurosci. Methods* **1993**, *48*, 225–240.
129. Koshland, D. E., Jr. *Science* **1992**, *258*, 1861.

Downloaded by UNIV OF GUELPH LIBRARY on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch011

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Chapter 12

Blue Native PAGE and Mass Spectrometry as an Approach for the Investigation of Stable and Transient Protein-Protein Interactions

Alisa G. Woods,[1,2] Izabela Sokolowska,[1,2] Rama Yakubu,[1]
Melissa Butkiewicz,[1] Martin LaFleur,[1] Christopher Talbot,[1]
and Costel C. Darie*,[1,2]

[1]Department of Chemistry & Biomolecular Science,
Biochemistry & Proteomics Group, Clarkson University,
8 Clarkson Avenue, Potsdam, New York, 13699-5810
[2]These authors contributed equally to this work
***Tel: (315) 268-7763. Fax: (315) 268-6610. E-mail: cdarie@clarkson.edu.**

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a relatively new method that separates protein complexes under native conditions, due to the external charge induced by Coomassie dye and according to their molecular weight (Mw). Colorless Native PAGE is a method similar to BN-PAGE, except that Coomassie dye is not used and therefore, separation of protein complexes takes place according to the internal charge of the protein complexes. Combination of BN-PAGE with other electrophoresis techniques or other biochemical approaches makes BN-PAGE a very powerful and versatile tool for the investigation of protein complexes. Furthermore, the combination of BN-PAGE with mass spectrometry (MS) is ideal for the structural and functional characterization of stable and transient protein-protein interactions.

Oxidative stress is a cellular response that is produced as a reaction to the generation of reactive oxygen species (ROS). These ROS have the potential to cause cellular and tissue damage, including changes in a cell's proteome. Oxidative stress can be investigated using MS at many levels: peptide, protein, protein complex or proteome using MS. However, proteomic analysis of oxidative stress may be difficult at

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

both the protein identification and protein quantitation levels and particularly difficult at the protein-protein interaction level. Here we discuss various applications of BN-PAGE including the characterization of stable protein complexes, transient protein-protein interactions, the assembly of proteins into polymers and the disassembly of protein complexes into sub-complexes. Future directions for BN-PAGE in combination with MS, with a focus on oxidative stress, are also discussed.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Introduction

Proteomics is the study of the proteome. The proteome is defined as the proteins in a particular cell, subcellular compartment or organelle, tissue, organ, extracellular fluid (blood, urine, saliva, tears or spinal fluid), or whole organism. A proteomics experiment usually involves 1) sample fractionation (during which proteins are biochemically separated using their physio-chemical properties); 2) enzymatic digestion of proteins into peptides; 3) analysis of a peptide mixture by liquid chromatography-mass spectrometry (liquid chromatography combined with tandem mass spectrometry or LC-MS/MS); 4) a database search, analysis and interpretation; 5) data validation.

Oxidative stress is a cellular response that is produced as a reaction to the generation of reactive oxygen species (ROS). These ROS have the potential to cause cellular and tissue damage, including changes in a cell's proteome. Oxidative stress can be investigated using MS at many levels: peptide, protein, protein complex or proteome using MS. However, proteomic analysis of oxidative stress may be difficult at both the protein identification and protein quantitation levels and particularly difficult at the protein-protein interaction level.

One extensively used proteomics experiment involves sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as the method of choice for protein fractionation. SDS-PAGE separates proteins under reducing and denaturing conditions and according to their Mw. A variation of SDS-PAGE is two dimensional (2D) SDS-PAGE or 2D-PAGE. This method separates proteins according to their isoelectric point (isoelectric focusing, IEF) in the first dimension (1D) and according to their Mw in the second dimension (2D). Other denaturing methods that are used in a proteomics experiment include SDS-PAGE in non-reducing conditions, (which maintains the intact disulfide-linked proteins), or Tricine PAGE, which separates proteins under denaturing conditions and provides a better resolution for the separation of low Mw proteins. Gel electrophoresis under native conditions is yet another method to produce protein fractionation prior to LC-MS/MS analysis. Among all of these methods, two types really stand out: Blue Native PAGE (BN-PAGE) and Colorless Native PAGE (CN-PAGE). These methods are easy to use, effective, accurate and produce a wealth of information. Our current review will focus on application of these two methods in proteomics.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

BN-PAGE and CN-PAGE as Choices for the Investigation of Protein Complexes and Protein-Protein Interactions

BN-PAGE was developed in Germany by Schaegger and von Jagow (*1–3*) and has long been used to analyze protein complexes first from plant organelles (*4–8*) and then later in animals (*7–25*). This method separates protein complexes based on an external charge induced by Coomassie dye and according to their Mw. BN-PAGE can be used to separate protein complexes in the range of 100-1500 kDa, using the advantage of Coomassie dye, which gives proteins negative charges through non-covalent, hydrophobic interactions, causing them to migrate to the anode at neutral running pH. Specifically, the Coomassie dye will bind to the protein complexes, so even basic proteins, because of the dye bound to them, will migrate to the anode.

BN-PAGE was discovered by investigating mitochondrial protein complexes involved in the electron transport chain, where most ROS are produced and where a high number of proteins are damaged by oxidative stress. Therefore, the use of BN-PAGE to investigate the oxidative stress at the protein complex level is of high interest and adds a new dimension to the investigation of oxidative stress at the protein-protein interaction level.

BN-PAGE experiments may provide information about the size, number, subunit composition, stoichiometry and relative abundance of these protein complexes. Separation of protein complexes from the BN-PAGE lane in a second dimension SDS-PAGE may reveal the subunit composition of a particular protein complex, as well as the interacting partners of a particular protein. Compared with the previous methods used to study protein-protein interactions, there are several advantages of BN-PAGE for studying protein-protein interactions: 1) separation of the protein complexes takes place under native conditions, so even the transient interactions between proteins may be identified, 2) separation of protein complexes in the second dimension may reveal their subunit composition, 3) the method may analyze the association of proteins into protein complexes as a result of a stimulus; 4) BN-PAGE may confirm the results obtained by immunoprecipitation experiments (*26*). For example, if proteins A, B and C co-immunopurify, BN-PAGE will often distinguish between A-B-C, A-B, A-C, and B-C complexes. Furthermore, by combining BN-PAGE with mass spectrometry, both structural and functional information may be obtained (*19*, *21*, *26*, *27*). CN-PAGE was developed by the same group that developed BN-PAGE (*1–3*) and further modified by others (*4*). CN-PAGE uses exactly the same procedure as CN-PAGE, except that the cathode buffer does not contain the Coomassie dye. In addition, the sample buffer does not contain the Coomassie dye either. Therefore, separation of protein complexes is not based on the external charge induced by Coomassie dye; it is rather based on the internal charge of the protein complex, given by the charge of its subunits. A schematic of the principle of BN-PAGE and CN-PAGE, as well as the differences between the two methods is presented in Figure 1. However, the principle of separation of BN-PAGE and CN-PAGE is very different, even though the separation in both methods takes place under the same native conditions. If in BN-PAGE, separation of the protein complexes takes place according to their Mw, CN-PAGE does not separate protein complexes according to their Mw. As such, the applications of these two methods with two different separation principles do not exclude each other, but rather complement each other.

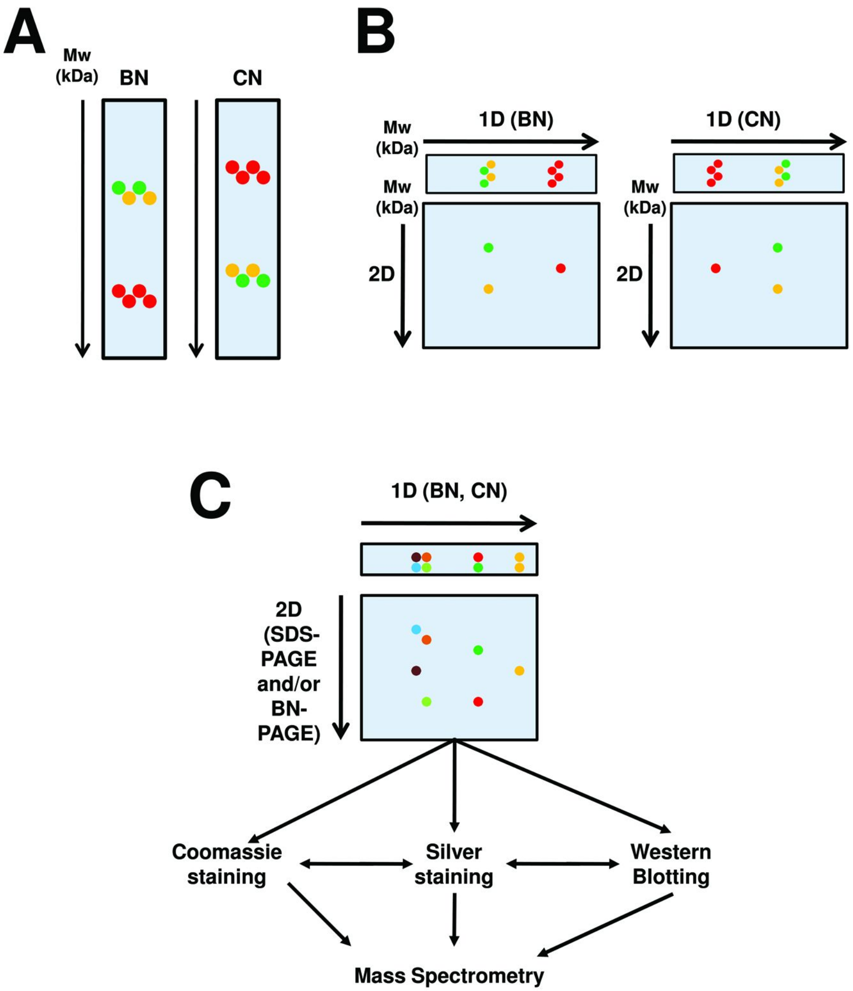

A
Mw (kDa)
BN
CN
B
1D (BN)
1D (CN)
Mw (kDa)
2D
C
1D (BN, CN)
2D (SDS-PAGE and/or BN-PAGE)
Coomassie staining
Silver staining
Western Blotting
Mass Spectrometry

Figure 1. ***BN-PAGE and CN-PAGE: Principles, similarities and differences.*** *Both BN-PAGE and CN-PAGE separate protein complexes under native conditions. BN-PAGE separates protein complexes due to the external charge induced by Coomassie dye (and according to the complexes' molecular mass). CN-PAGE separates protein complexes due to their internal charge (no Coomassie dye is used) and independent of complexes' molecular mass. Therefore, protein complexes separated by BN-PAGE may not separate the same in CN-PAGE (A). BN-PAGE and CN-PAGE may separate homo-protein complexes (the subunits are red) and hetero-protein complexes (the subunits are both yellow and green). The subunit composition of the protein complexes*

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

may be revealed by separation of the protein complexes in a second dimension by SDS-PAGE, under denaturing and reducing conditions (B). As such, the homo-protein complex will separate in one subunit (red subunit), while the hetero-protein complex will separate in two or more subunits (in this case two: yellow and green subunits). (C) The protein complexes that were separated by BN-PAGE or by CN-PAGE may be further separated by SDS-PAGE (as shown in (B)) or by BN-PAGE, followed by staining (by Coomassie, Silver, Zinc, Sypro, etc) and further analyzed by mass spectrometry (both MALDI-MS and LC-MS/MS). The gels from the second dimension may also be analyzed by Western blotting and further analyzed by mass spectrometry. These methods complement each other (e.g. COomassie and Silver staining or Silver staining and Western blotting).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Here we will show some examples of use of BN-PAGE either alone or in combination with other electrophoresis (e.g. CN-PAGE, Tricine PAGE, SDS-PAGE; see below) and mass spectrometry (e.g. MALDI-MS and LC-MS/MS, both mass spectrometry, MS; see below) techniques to either identify the Mw or subunit composition of a stable or transient protein complex of mammalian origin, or to isolate and characterize a protein complex of vegetal origin, with emphasis on oxidative stress and repair. Overall, we demonstrate that the combination of BN-PAGE and MS has the potential to be generally applied to the analysis of any stable and transient protein-protein interactions.

BN-PAGE as a Tool for Determination of the Mw of Protein Complexes and for Determination of Transient Protein-Protein Interactions

Receptor tyrosine kinases (RTK) are receptors for a series of growth factors that are important for cell survival and differentiation during development as well as in response to disease or injury (*28*, *29*). A common feature for these RTKs is that their growth factor ligands are typically secreted proteins. Eph receptors (EphRs) represent a different class of RTKs and are the largest sub-family of RTKs (*30*). These EphRs are found in a variety of cell types from developing and from mature tissues and play important roles in the development of the central and peripheral nervous systems (*31–34*), including synapse formation (*35*) and neural crest formation (*36*). They also play roles in the vascular system, including angiogenesis and vascular system remodeling (*37–39*).

EphRs differ from other RTKs in three regards: 1) EphRs do not promote cell growth, survival and differentiation, but instead play important roles in the attractive and repellent reactions between cells (*40*). 2) Activation of EphRs is performed by ephrins that, unlike the other soluble RTKs ligands, are anchored to the cell membrane. 3) During stimulation, EphRs may be either receptors that are activated by ephrin ligands and trigger downstream signaling or they may be ligands that activate upstream signaling in ephrin-bearing cells. To date, at least 14 different EphRs have been identified and based on the type of the ligand with which they interact, they have been divided into two classes: EphAs and EphBs (*41*). EphA receptors comprise nine members: EphA1-A8 and EphA10. They interact with ephrins A which consist of five members: ephrinA1-A5. EphB receptors comprise five members: EphB1-B4 and EphB6. They interact with ephrins B which consist of three members: ephrinB1-B3 (*41*). Although all EphRs and ephrinBs are transmembrane proteins that contain extracellular and cytosolic domains, ephrinAs are connected to the cell surface by glycosylphosphatidylinositol anchors, and therefore do not contain a cytosolic domain.

Interaction of ephrins with Eph receptors leads to a gradual formation of ephrin-EphRs multimers that trigger cytoskeletal remodeling. This underlies cell adhesion and motility in ligand and receptor bearing cells (*41–44*). The first consequence of EphR activation is autophosphorylation of its three conserved tyrosine residues from the cytoplasmic side of the receptor. The phosphorylated sites provide docking for a number of SH2-domain containing signaling and adaptor proteins that modulate cytoskeletal plasticity (*42–44*).

A number of proteins that interact with phosphorylated EphRs or mediate downstream signaling have been identified. For example, Ephexin, a member of the Rho family of GTPases from retinal ganglion cells, is constitutively associated with EphA receptors. Ephexin activates RhoA and inhibits Rac1 and Cdc42 by switching the actin cytoskeleton from an extended to a contracted state (*44*, *45*). Other proteins that interact directly or indirectly with EphRs or that have been identified as a component of EphR downstream signaling include: Nck, Ras GTPase-activating protein, p85-associated phosphoinositide 3-kinase, SLAP, Grb2, p62dok (*31*, *46–53*). Recently, a clearer dissection of the EphB2 signaling from the unstimulated and ephrinB1-stimulated cells has been performed using Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) technology (*54*, *55*).

The first step in investigating ephrin signaling is to identify stable, constitutively expressed protein complexes, both with identical subunit composition (homo-complexes) and different subunit composition (hetero-protein complexes). To do so, NG108 (mouse neuroblastoma x rat glioma) cells were grown *in vitro* as previously reported (*25*) and stimulated with ephrinB1-Fc, then lysed. Unstimulated cells were used as controls. A schematic of the experimental procedure is shown in Figure 2A. The cell lysates were then separated by BN-PAGE 1D and then Coomassie or silver stained (Figure 2B), or further reduced and denatured and separated in SDS-PAGE 2D, followed by silver staining (Figure 2C) or Western blotting (WB, Figure 2D). As observed, in the silver-stained BN-PAGE 1D gel lanes, we could observe intense bands that corresponded to protein complexes with a mass of about 750, 670, 600, 440 and 400 kDa, suggesting that BN-PAGE is a good tool for identification of the Mw of protein complexes. The use of WB in combination with BN-PAGE may also allow us to identify transient protein-protein interactions. As observed, in the WB experiments with anti-phosphotyrosine antibodies, we could demonstrate that 1) stimulation of NG108 cells *in vitro* by the ephrinB1-Fc ligand is effective and activates the EphB2-dependent signal transduction pathway and 2) BN-PAGE 2D, when combined with WB (Figure 2D), is an excellent tool for detection of transient protein-protein interactions.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

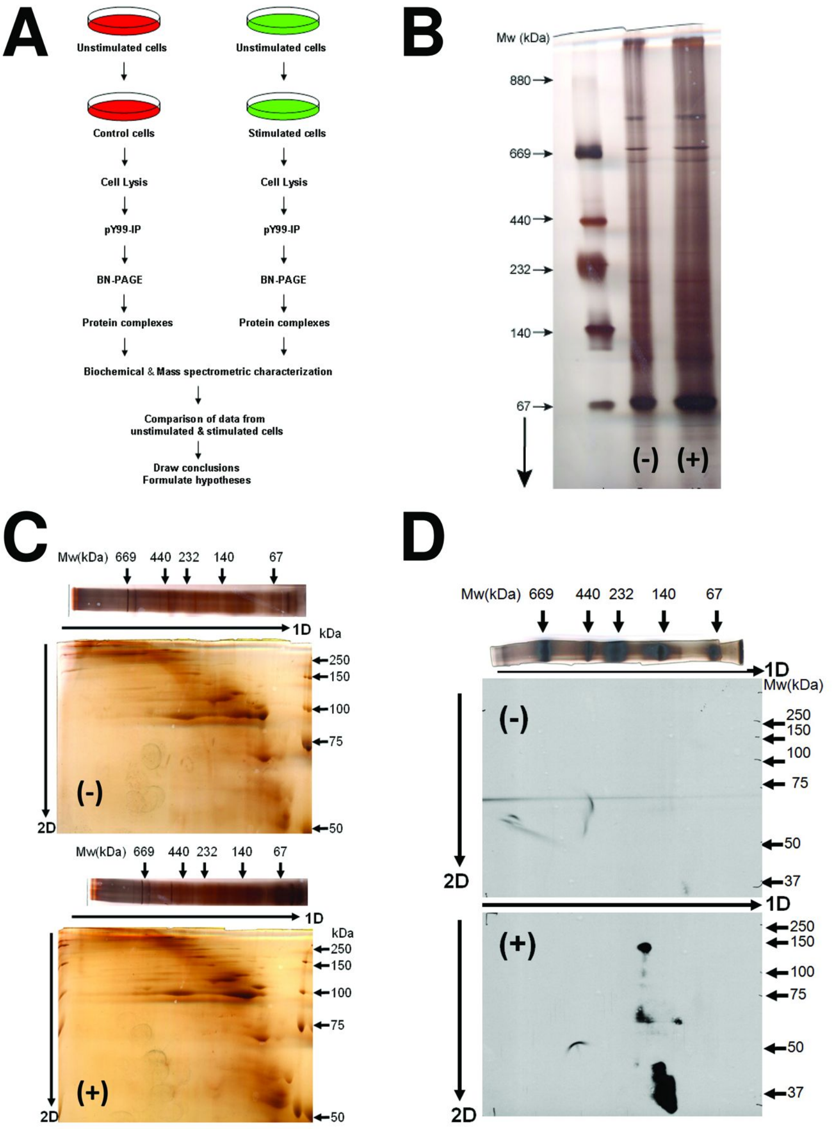

A
Unstimulated cells
Unstimulated cells
Control cells
Stimulated cells
Cell Lysis
Cell Lysis
pY99-IP
pY99-IP
BN-PAGE
BN-PAGE
Protein complexes
Protein complexes
Biochemical & Mass spectrometric characterization
Comparison of data from unstimulated & stimulated cells
Draw conclusions
Formulate hypotheses
B
Mw (kDa)
880
669
440
232
140
67
(-)
(+)
C
Mw(kDa) 669 440 232 140 67
1D
kDa
250
150
100
75
50
2D
(-)
Mw(kDa) 669 440 232 140 67
1D
kDa
250
150
100
75
50
2D
(+)
D
Mw(kDa) 669 440 232 140 67
1D
Mw(kDa)
250
150
100
75
50
37
2D
(-)
1D
250
150
100
75
50
37
2D
(+)

Figure 2. (A): ***A workflow for the biochemical and MS characterization of ephrin signaling.*** *The unstimulated or stimulated cells were lysed and the lysates (and/or the pY99-IPs from the lysate) were separated by BN-PAGE. The protein complexes/protein interactions were characterized biochemically and/or by MS, followed by comparison with the current literature. (B): Silver stain of BN-PAGE 1D of the cell lysate from unstimulated (-) and ephrinB1-Fc stimulated cells (+). The direction of migration in gel and Mw markers are indicated. (C): BN-PAGE 2D: the cell lysates of the unstimulated (-) and stimulated (+) cells were separated by BN-PAGE 1D as in (B) and then reduced and denatured and further separated by denaturing SDS-PAGE followed by silver staining. The* *direction of migration in the gel and Mw markers are indicated. Note the high Mw markers in BN-PAGE 1D (for protein complexes) and normal Mw markers in SDS-PAGE 2D (for proteins). (D): BN-PAGE 2D: the experiment is as in (C), except that the gels from SDS-PAGE 2D, instead of being silver stained, were electroblotted and analyzed for the presence of tyrosine phosphorylated proteins and protein complexes by using anti-phosphotyrosine antibodies.*

BN-PAGE and MS as a Tool for Determination of the Subunit Composition of Stable Homo- and Hetero-Protein Complexes

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

We further investigated whether intact, constitutively expressed protein complexes from the lysates of unstimulated and stimulated cells may be identified by BN-PAGE and LC-MS/MS experiments at a molecular weight similar to the one predicted by their subunit composition and in accordance with published work. In the silver-stained (Figure 2B) and Coomassie-stained (data not shown) BN-PAGE 1D gel lanes we could observe putative protein complexes with masses ranging from about 750 to about 400 kDa. To reveal the protein composition of some of these complexes, we cut out the most intense gel bands that corresponded to protein complexes with molecular masses between 400 and 750 kDa, digested them with trypsin and analyzed them by LC-MS/MS. When we identified a protein that was known to be a subunit of a particular protein complex, we looked for additional subunits in the data that resulted from the same LC-MS/MS experiment and further compared these data with the current literature in regard to subunit composition, molecular mass, protein interactions, etc. In our BN-PAGE and LC-MS/MS experiments, we identified protein complexes that are known to be constitutively expressed in many mammalian cells. Their subunit composition was either a multimer of one subunit only (homo-protein complexes) or multimer of two or more subunits (hetero-protein complexes).

The homo-protein complexes identified by BN-PAGE and LC-MS/MS included Valosin-containing protein and ATP citrate lyase. Valosin-containing protein, also named Transitional endoplasmic reticulum ATPase is a 89 kDa protein which, under native conditions, is a homohexamer. ATP citrate lyase is a 120 kDa protein that under physiological conditions is a homotetramer. Valosin-containing protein is involved in the formation of the transitional endoplasmic reticulum, specifically in the transfer of membranes from the endoplasmic reticulum to the Golgi apparatus. It is also required for the fragmentation of Golgi stacks during mitosis and for their reassembly at the end of mitosis (*56*). ATP citrate lyase is responsible for the synthesis of cytosolic Acetyl-COA (*57*, *58*). The theoretical Mw of the Valosin-containing protein complex is 540 kDa, while the theoretical Mw of ATP citrate lyase is 480 kDa. We found these protein complexes in gel bands that corresponded to masses of 550-650 kDa. This is in agreement with a report that the 89 kDa Valosin-containing protein is usually a homohexamer and the 120 kDa ATP citrate lyase protein complex is a homotetramer. An example of MS and MSMS spectra of an identified peptide that is part of the 120 kDa ATP citrate lyase protein (that was a subunit of the 480 kDa homocomplex composed of four identical subunits) is shown in Figure 3.

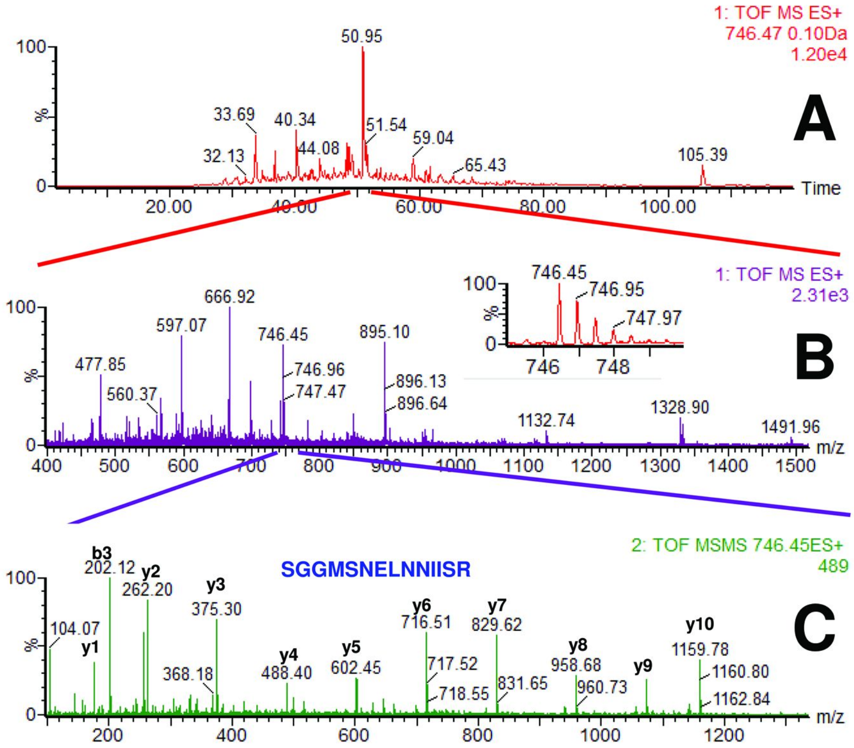


Figure 3. ***Analysis of a homo-protein complex by BN-PAGE and LC-MS/MS.*** *The gel band was excised and digested by trypsin and the resulting peptide mixture was analyzed by LC-MS/MS. The extracted ion chromatogram (EIC) of the peak with m/z of 746.47 identified in the chromatogram a peak at 50.95 minutes (A) that contained MS spectra of a double charged (2+) peak with m/z of 746.45 (B). The isotopic resolution of this peak is shown in inbox (B). This peak was selected for fragmentation by MSMS (C) and produced a series of b and y fragment ions. Data processing and database search led to the identification of a peptide with the sequence SGGMSNELNNIISR that is part of ATP citrate lyase.*

In our experiments using BN-PAGE and LC-MS/MS, we also identified many hetero-protein complexes. One of them was proteasome (prosome), a protein complex involved in protein degradation (*59–67*). Proteasome is hetero-protein complex that contains 28 protein subunits. It is involved in protein degradation and is composed of seven alpha (mass 27 kDa) and seven beta (mass 29 kDa) subunits contained in a hetero-28-mer. LC-MS/MS experiments of the BN-PAGE bands allowed us to identify five different subunits alpha (alpha 1, alpha 3, alpha 4, alpha 6 and alpha 7) and four different subunits beta (beta 1, beta 4, beta 5 and beta 6) in a single experiment (data not shown). The predicted Mw of this complex

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

is about 700 kDa. In our experiments, we identified this complex in the same 700 kDa range, suggesting that BN-PAGE may provide information about the Mw of a protein complex.

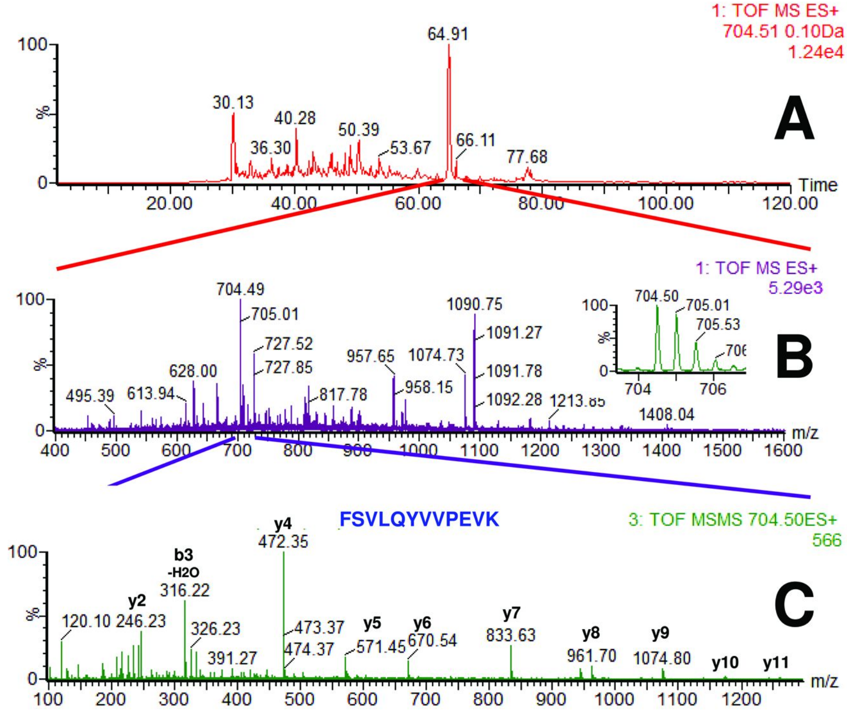


Figure 4. ***Analysis of sub-complex 1 of the eukaryotic initiation factor 3 hetero-protein complex by BN-PAGE and LC-MS/MS.*** *The gel band was excised and digested by trypsin and the resulting peptide mixture was analyzed by LC-MS/MS. The extracted ion chromatogram (EIC) of the peak with m/z of 704.51 identified in the chromatogram a peak at 64.91 minutes (A) that contained MS spectra of a (2+) peak with m/z of 704.49 (B). The isotopic resolution of this peak is shown in inbox (B). This peak was selected for fragmentation by MSMS (C) and produced a series of b and y fragment ions. Data processing and database search led to the identification of a peptide with the sequence FSVLQYVVPEVK that is part of the subunit 10 of the eukaryotic initiation factor 3 (eif3S10), which is part of sub-complex 1 of eukaryotic initiation factor 3 complex.*

Another heteroprotein complex that we identified was eukaryotic translation initiation factor 3 complex (eIF3) that plays important roles in protein synthesis. The eIF3 complex is composed of 13 subunits: EIF3A through M. Through a different nomenclature, they are named Eif3-S1 (S from subunit) through Eif3-S10, Eif3-S12, Eif3-S6-interacting protein and Ga17 (also named Pcid1). The eIF-3 complex contains three subcomplexes. Sub-complex one is composed of EIF3A, B, G and I; the second one contains EIF3F, H, and M, and the third one contains EIF3C, D, E, K and L. EIF3C of sub-complex 3 binds EIF3B of sub-complex 1 and EIF3H of sub-complex 2, thereby linking the subcomplexes together. The calculated Mw of this complex is about 800 kDa (*68–72*). In our BN-PAGE and LC-MS/MS experiments, we identified the eIF3 at the predicted Mw of about 800 kDa. Of the 13 total subunits, we identified at least 10 different subunits that were part of subcomplexes 1, 2 or 3 of the eIF3, suggesting that the eIF3 complex is intact and contains all three eIF3 protein subcomplexes. An example of MS and MSMS spectra of a peptide that was identified and that was part of eIF3 complex is shown in Figure 4. Taken together, these data suggest that the combination of BN-PAGE and LC-MS/MS is a powerful tool for determining the mass of a particular protein complex and the identity of its subunit composition.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

BN-PAGE and WB as a Tool for Identification of Transient Protein-Protein Interactions

In addition to the determination of the Mw and subunit composition of stable homo- and hetero-protein complexes, BN-PAGE and LC-MS/MS is also able to identify transient protein-protein interactions. To identify these transient protein-protein interactions, the protein complexes from the unstimulated and ephrinB1-Fc-stimulated NG108 cells were immunoaffinity purified using anti-phosphotyrosine (pY99-IP) antibodies and then separated by BN-PAGE. The BN-PAGE gel lanes that contained protein complexes were cut in gel pieces according to their molecular weight and then subjected to trypsin digestion. The resulting peptide mixtures were then analyzed by LC-MS/MS, and the resulting data were submitted to the Mascot database for protein identification. These experiments provided a list of proteins for each gel band that may be part of the same protein complex. The list of proteins for each gel piece was compared with the lists of proteins from the other gel pieces from the same condition (either unstimulated cells or stimulated cells) and then between the unstimulated and stimulated cells. Comparison of these lists of proteins, as well as construction of the interaction networks between the signaling proteins, was performed by reconstituting the original gel and by comparing the data with the current literature. The data were interpreted based on the simplest theoretical scenarios that may happen with a particular protein, presented in Figure 5, complemented by the current literature. A full list of the protein complexes and the transient protein-protein interactions that were identified by BN-PAGE and LC-MS/MS is currently under review (data not shown).

A way to further demonstrate that BN-PAGE is suitable for the analysis and identification of transient protein-protein interactions following ligand stimulation is to combine BN-PAGE and WB. This allows the monitoring of transient interactions of a single protein using antibodies against that protein. Therefore, we analyzed cell lysates of mouse cortical neurons that were stimulated by a different ligand, brain-derived neurotrophic factor (BDNF) that binds to Trk receptors, and focused on one particular protein, Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs, mass 115 kDa). The Hrs protein is involved in endocytic processes (*25*, *73–75*). To do so, we separated the cell lysates of neurons that were treated or not treated with BDNF by BN-PAGE 1D, SDS-PAGE 2D and then electroblotted the proteins onto a membrane and incubated them with anti-HRS antibodies, followed by enhanced chemiluminescence detection. As may be seen in Figure 6, the Hrs antibodies recognized a band at about 200 kDa in unstimulated cells and two bands at 200 kDa and 500 kDa in stimulated cells. The bands at about 200 kDa is the Hrs monomer (115 kDa), either alone or in interaction with a small Mw protein. However the band at 500 kDa represents Hrs in complex with many proteins as a result of BDNF stimulation. Therefore, the 500 kDa band detected the transient interactions of Hrs with other proteins. Taken together, these experiments suggest that, regardless of the cell system, as long as there are two different experimental conditions, they may be compared by BN-PAGE and the differences between the two states may be determined by BN-PAGE and WB and identified by BN-PAGE and LC-MS/MS.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

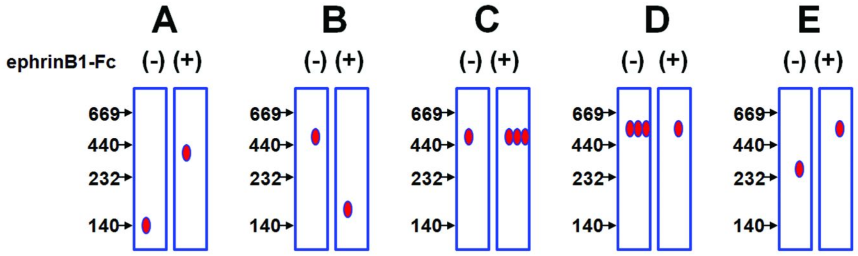


Figure 5. ***Possible theoretical scenarios for the analysis of protein-protein interactions using a pY99-IP.*** *A: One protein interacts with other proteins upon stimulation and forms a protein complex with a higher molecular mass; at least one subunit of this newly formed complex is phosphorylated at a tyrosine residue. B: One protein complex is disassembled upon stimulation. C: One protein complex does not have any changes in its subunit composition, but one or more of its subunits become over-phosphorylated at a tyrosine residue upon stimulation. D: One or more subunits from one super-phosphorylated protein complex are de-phosphorylated at a tyrosine residue upon ephrinB1-Fc stimulation. E: A protein complex is completely de-phosphorylated (300 kDa) or becomes phosphorylated (600 kDa) upon ephrinB1-Fc stimulation.*

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

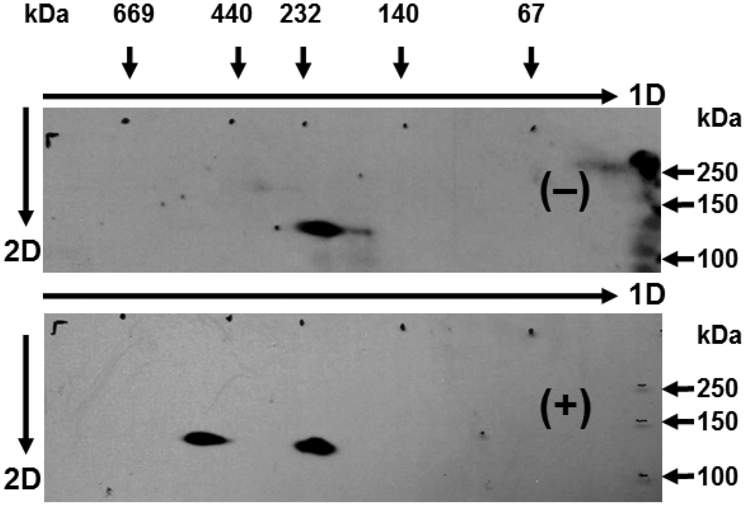


Figure 6. ***Identification of transient protein-protein interactions in primary neurons by BN-PAGE and WB.*** *Analysis of the lysates of unstimulated (–) and BDNF-stimulated (+) was performed using primary neuronal cell cultures by BN-PAGE. The lysates were separated in BN-PAGE 1D and SDS-PAGE 2D, and electroblotted, and then analyzed for the presence of Hrs protein using anti-Hrs antibodies. The immune reaction was visualized using enhanced chemiluminescent reaction. As observed, Hrs forms a 500 kDa protein complex upon BDNF stimulation.*

BN-PAGE as a Tool in Analysis of Protein Self-Assembly into Higher Mw Polymers

Another application of BN-PAGE is the analysis of the polymerization state of one or more proteins. As an example, polymerization of vitelline envelope proteins was investigated by BN-PAGE and further confirmed by electron microscopy. The plasma membrane of vertebrate eggs is surrounded by an extracellular envelope, named vitelline envelope (VE) in fish and zona pellucida (ZP) in mammals. VE is composed of three proteins: VE alpha (58 kDa), VE beta (52 kDa) and VE gamma (47 kDa). These proteins have a common domain (about 260 amino acids long) named the zona pellucida (ZP) domain. The ZP domain is responsible for the polymerization of proteins into higher order structures, such as fibrils and filaments (*23*, *24*, *76–82*). However, prior to our experiments the mechanism of polymerization was not known. To reveal the mechanism of polymerization of the ZP domain-containing proteins, we purified VE beta and VE gamma and analyzed how the individual VE proteins and the combination of both assemble into high Mw polymers and filaments. To do so, we analyzed individual VE proteins by BN-PAGE and observed that, in the presence of denaturant, they form higher Mw polymers (starting with homo-dimers). When different VE proteins were mixed and then analyzed by BN-PAGE,we could observe that these proteins polymerized into higher order structures (*23*). These finding were further confirmed by electron microscopy, where the fully assembled/polymerized VE proteins were observed as high Mw filaments. Therefore, BN-PAGE is a useful tool for the analysis of the polymerization state of a protein or mixture of proteins.

BN-PAGE as a Tool for the Determination of the Mw and Multimerization State of a Stable Protein Complex Protective against Oxidative Stress

So far, BN-PAGE is one of the fastest and cheapest ways to determine the Mw of a protein complex, especially when dealing with complex samples like cell lysates or bodily fluids (e.g. blood). This is particularly important when one tries to identify the Mw and subunit composition of a protein complex, as well as the sub-complexes and super-complexes of that protein complex. This property lies within its principle: it separates protein complexes due to the external charge induced by Coomassie dye and according to their Mw. An example of how separation by BN-PAGE can be used for the determination of the Mw of the complex, the subunit composition of its sub-complexes and the polymerization state of the protein complex is shown in Figure 7. Here, thylakoid membranes from mesophyll chloroplasts of maize were separated by BN-PAGE for identification of the Mw and subunit composition of NAD(P)H-dehydrogenase (Ndh) complex. Maize is a C4-type plant and contains two types of plastids: mesophyll and bundle sheath chloroplasts. Ndh complex is a plastidal complex involved in chlororespiration, which is homologue to mitochondria complex I (*4*, *83–91*). Separation of the thylakoid membranes on BN-PAGE in 1D led to apparition of gel bands that correspond to protein complexes of a particular Mw (Figure 7A). Separation of the BN-PAGE gel lanes in a second Tricine-PAGE 2D (under denaturing and reducing conditions) followed by Coomassie (data not shown) or silver staining (Figure 7A) led to disintegration of the protein complexes from 1D into their subunits in 2D. Tricine PAGE is a gel electrophoresis method similar to SDS-PAGE, but which has a better resolution in separation of the lower Mw proteins.

Once the thylakoid membranes were separated by BN-PAGE 1D and Tricine PAGE 2D, the gel was further electroblotted (WB) and analyzed using antibodies against proteins that were known to be subunits of one protein complex. Figures 7B and C show the WB of the BN-PAGE 2D that was analyzed with antibodies against subunits of Ndh complex, to monitor the integrity of the Ndh complex and its multimerization state (e.g. monomer or dimer). The Ndh complex contains a membrane sub-complex (300 kDa) and a soluble sub-complex (250 kDa) and the antibodies that were used were against protein subunits of the Ndh complex which were part of the membrane sub-complex (Ndh E, 12 kDa) or against the soluble subcomplex (NdhH, 45 kDa; NdhJ, 21 kDa; NdhK, 29 kDa). As observed, the Mw of the intact Ndh complex is 550 kDa, as determined by the antibodies against all four protein subunits, which reacted with their antigens (both part of membrane and soluble sub-complexes (Figure 7B), in agreement with the Mw reported by others. In addition, the Ndh complex splits into the membrane (300

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

kDa, detected by NdhE) and soluble (250 kDa, detected by NdhJ & NdhK) subcomplexes (Figure 7B), and exists as monomeric and dimeric forms (Figure 7C, (*4*)).

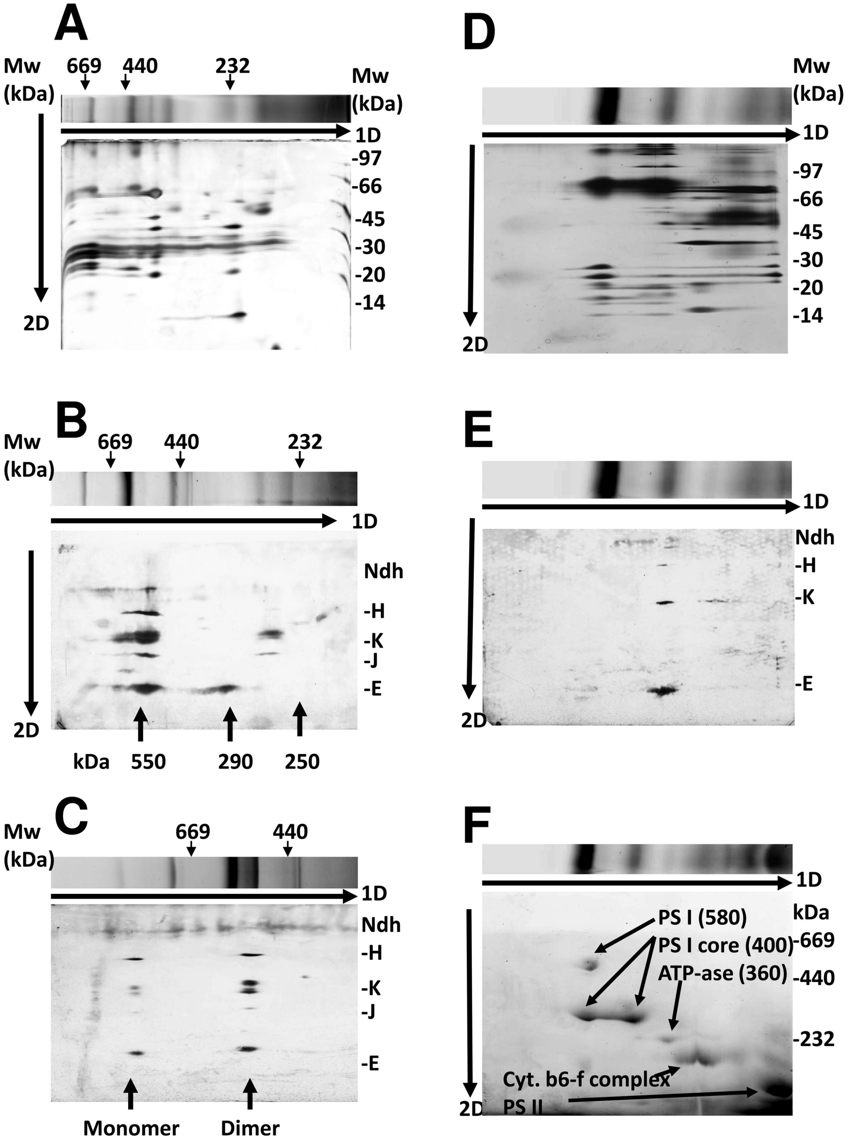

A
Mw (kDa)
669
440
232
Mw (kDa)
1D
-97
-66
-45
-30
-20
-14
2D
B
Mw (kDa)
669
440
232
1D
Ndh
-H
-K
-J
-E
2D
kDa
550
290
250
C
Mw (kDa)
669
440
1D
Ndh
-H
-K
-J
-E
Monomer
Dimer
D
Mw (kDa)
1D
-97
-66
-45
-30
-20
-14
2D
E
1D
Ndh
-H
-K
-E
2D
F
1D
kDa
PS I (580)
PS I core (400)
ATP-ase (360)
-669
-440
-232
Cyt. b6-f complex
PS II
2D

*Figure 7. **Structural characterization of the Ndh complex.** The thylakoid membranes were separated by BN-PAGE 1D and Tricine PAGE 2D and then silver stained (A) or electroblotted and analyzed with Ndh antibodies (B, C). The thylakoid membranes were also separated by CN-PAGE 1D and Tricine PAGE 2D and the silver stained (D) or electroblotted and analyzed with Ndh antibodies (E). After separation by CN-PAGE 1D, the protein complexes were further*

separated by BN-PAGE 2D and stained by Coomassie (F). Location of PS I, ATP-ase, Cyt b6f complex and PS II is indicated. The Mw markers for BN-PAGE and Tricine PAGE are shown. There are no Mw markers for CN-PAGE, because this method does not separate protein complexes according to their Mw. The direction of migration in BN-PAGE, Tricine PAGE and CN-PAGE is indicated.

CN-PAGE as a Pre-Purification Tool for the Localization of a Stable Protein Complex Protective against Oxidative Stress in BN-PAGE

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

The main goal of this project was to isolate and characterize the Ndh complex. However, as observed in Figure 7A, the Ndh complex is not visible in the BN-PAGE 1D nor in Tricine-PAGE 2D. Therefore, although BN-PAGE can be very useful in the fast isolation and characterization of a protein complex, if in BN-PAGE the Mw of the protein complex of interest (Ndh complex) has the same Mw as a different complex, then an additional purification procedure prior to BN-PAGE is required. CN-PAGE is such a procedure. It separates protein complexes according to their internal charge. Figure 7 shows the CN-PAGE 1D and Tricine-PAGE 2D of the thylakoid membranes from mesophyll chloroplasts, followed by silver staining (Figure 7D) or WB (Figure 7E) using antibodies against NdhE (monitors the 300 kDa membrane protein sub-complex) and NdhK (monitors the 250 kDa soluble protein sub-complex). As observed, the intact Ndh complex is localized in the second most intense band of the CN-PAGE 1D (Figure 7E).

Since the WB of CN-PAGE 1D and Tricine PAGE 2D (Figure 7E) allowed us to immuno-localize the Ndh complex in CN-PAGE 1D (the second most intense band), we continued our purification and further separated the gel lane from CN-PAGE 1D in BN-PAGE 2D (Figure 7F). This separation allowed us to better localize the Ndh complex in the BN-PAGE, information that will be used for further characterization. In Figure 7F, the protein complexes are seen as dark spots and, based on mass spectrometry data (not shown), they include Photosystem I (PSI, 580 & 400 kDa spots), ATP-synthase complex (360 kDa spot), Cytochrome b6f complex (230 kDa) and part of Photosystem II (PSII, 210 kDa). As observed, the Ndh complex is not visible. However, since we knew that the Ndh complex has a Mw of 550 kDa and it is part of (or close to) the second band in the CN-PAGE, then we could localize it within a square formed by the 580 kDa PSI and the two 400 kDa PSI core. It should be noted that the two 400 kDa PSI complexes have an identical Mw, but only their internal charge is different, most likely due to post-translational modifications such as phosphorylations. Therefore, the combination of CN-PAGE and BN-PAGE is able to separate two protein complexes with identical Mw, but different internal charge.

To identify the exact location of the Ndh and for its further characterization, we cut horizontal and vertical gel bands from the above mentioned square, known to contain the Ndh complex and separated them in a SDS-PAGE 3D, followed by WB using antibodies against Ndh subunits (Figure 8A). As observed, the position of the Ndh complex in WB of the SDS-PAGE was localized in the second band in both horizontal and vertical gel bands (Figure 8A). To confirm the localization of the Ndh complex, we 1) superimposed the horizontal and vertical BN-PAGE 2D gel bands that were analyzed by WB (and shown in Figure 8A); 2) silver stained the BN-PAGE 2D gel that contained the square formed by the 580 kDa PSI and the two 400 kDa PSI core (Figure 8B); 3) electroblotted the BN-PAGE 2D gel under native conditions and analyzed it with Ndh antibodies (Figure 8B). As observed, the position in the gel of the Ndh complex localized by WB of the SDS-PAGE 3D gels was confirmed by both silver staining and WB of the BN-PAGE 2D (Figure 8B).

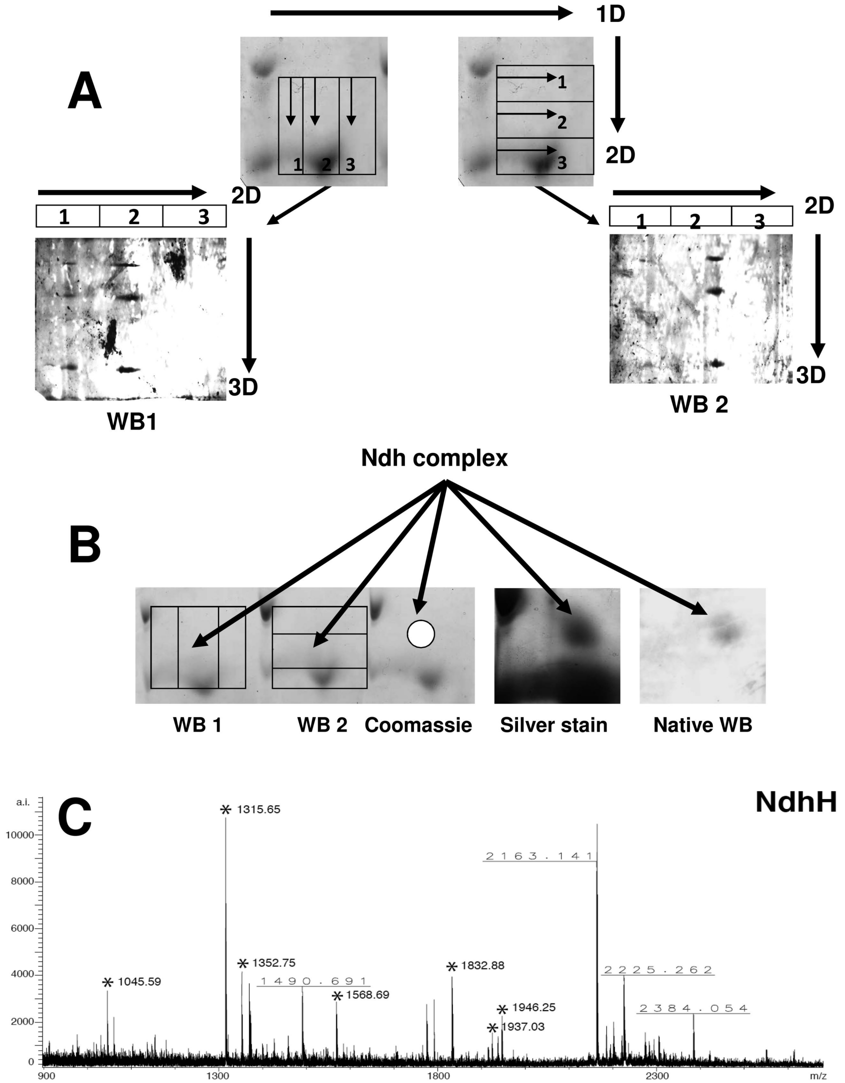


Figure 8. ***Localization of the Ndh complex in BN-PAGE PAGE 2D and its characterization by MALDI MS.*** *The Ndh complex was separated by CN-PAGE*

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

and BN-PAGE and localized in BN-PAGE 2D in the square containing intact PS I and PS I core complexes (previous experiments from Figure 9). Horizontal and vertical gel bands from BN-PAGE 2D were separated by Tricine PAGE 3D and analyzed by WB using anti-Ndh antibodies. The vertical gel bands were analyzed by WB1 and the horizontal gel bands were analyzed by WB2 (A). The position of the Ndh complex in the BN-PAGE 2D gel, as determined by WB1 and WB2 (from A) is shown in (B). The expected location of the Ndh complex, based on the superimposition of WB1 and WB2 is shown in the gel stained with Coomassie; no band/spot was observed in that location in the Coomassie stained gel. Different BN-PAGE 2D gels were analyzed for the exact location of the Ndh complex (here shown only the square containing the PS I and PS I core complexes) by silver staining (B) or electroblotted and analyzed with anti-Ndh antibodies (native WB, B). The gel spot containing the Ndh complex (localized by the experiments from (A) and (B)) was excised and separated by Tricine-PAGE (not shown), digested and analyzed by MALDI-MS (C). Data processing and database search led to identification of some subunits of the Ndh complex. The MALDI-MS spectrum that identified NdhH, a subunit of the Ndh complex is shown in (C).

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

CN-PAGE 1D, BN-PAGE 2D and SDS-PAGE 3D may be combined with Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) for the determination of the subunit composition of a protein complex. To further characterize the Ndh complex, we performed a preparative CN-PAGE 1D and BN-PAGE 2D, excised the gel band that contained the Ndh complex, and separated it by SDS-PAGE 3D, followed by silver staining. The most intensely stained gel bands were excised, digested with trypsin and analyzed by MALDI-MS. Figure 8C shows an example of a MALDI-MS spectrum of the SDS-PAGE 3D. Data processing and database search led to the identification of NdhH, a 45 kDa protein that is a subunit of the Ndh complex. Therefore, in addition to being compatible with LC-MS/MS, BN-PAGE is also compatible with MALDI-MS.

BN-PAGE and Mass Spectrometry as Methods for Examining Oxidative Stress

Initially, BN-PAGE was discovered using mitochondrial protein complexes, the location at which the greatest amounts of oxidative species appear. However, within the first ten years since its discovery (1991-2001), the use of BN-PAGE has been limited to plant scientists. Furthermore, this method has not been used in combination with any other mass spectrometry-based approaches. A simple literature search using BN-PAGE as a keyword revealed 476 hits, of which 61 studies were performed since its discovery (1991) until 2000. The remaining 415 studies were performed within the past 11 years, demonstrating the growing significance of this method. A literature search using BN-PAGE and mass spectrometry as keywords revealed 127 studies, and in only four studies the authors combined BN-PAGE with mass spectrometry. However, this number grew considerably within the past ten years (123 studies), and BN-PAGE has been used in combination with both MALDI-MS or LC-MS/MS or both. Examples of representative work include, but are not limited to, work performed in cell organelle, tissues, organs or organisms from plants, animals or bacteria (*21*, *92–98*).

The main application of BN-PAGE, either alone or in combination with other methods was determination of the molecular mass of a protein complex and of its subunit composition. However, recently, its applications became very diverse and range from the determination of the polymerization state of a protein or protein complex, to the identification of transient protein-protein interactions and post-translational modifications of the subunits of a protein complex. In fact, identification of transient protein-protein interactions is perhaps one of the most important applications for BN-PAGE and, when combined with Differential Gel Electrophoresis (DIGE) and mass spectrometry (*16*, *99*, *100*), it becomes a very powerful analytical tool. Despite that, BN-PAGE is not the most frequently used tool for the examination of oxidative stress. A literature search revealed that there are very few studies utilizing BN-PAGE in the examination of oxidative stress. In one study, Andriga et al. (*10*) described the use of one thiol-labeling approach in combination with blue native gel electrophoresis (BN-PAGE) to detect reactive thiol groups within mitochondrial proteins, with a focus on the protein complexes involved in oxidative phosphorylation. Kim et al. (*101*) focused on the analysis of proteins and their post-translational modifications to identify the age-related differences in protein abundance and isoform complexity. This group also tried BN-PAGE and concluded that the oxidative stress and altered protein interactions may be at the heart of age-related diseases and therefore oxidative protein modifications and protein-protein interactions have special relevance to age-related research. Additional studies focused on monitoring protein complexes from plant mitochondrial respiratory oxidative phosphorylation (*102*, *103*), as well as mitochondrial NADH dehydrogenase or Complex I (homologue of the chloroplastic Ndh complex) from rodent brain/dopaminergic neuronal cell line (*104*), and human neuroblastoma cells (*105*). Although these studies had a diverse range of starting material, the end goal was the same: to identify the reasons for oxidative stress at the protein complex level.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Limitations of BN-PAGE.

As demonstrated here through quite a few examples, BN-PAGE has a wide variety of applications. However, one should be aware that this method also has limitations. One of these limitations is due to the isoelectric point of protein complexes; if their isoelectric point is below 5.4 or above 8.6, the accuracy of this method in the determination of the molecular masses of protein complexes does not apply anymore. In addition, depending on the subunit composition of protein complexes, determination of the molecular mass of these complexes is not very accurate, sometimes with a deviation of up to 10-15% (data not shown). Furthermore, simultaneous analysis of multiple transient protein-protein interactions by BN-PAGE and mass spectrometry, although possible, is still very difficult to achieve. This is mostly because more than one protein complex may have identical or similar molecular mass and the subunits of one complex may be erroneously assigned to a different protein complex.

Concluding Remarks

Overall, BN-PAGE opens new possibilities for simultaneously analyzing the whole proteome at the protein complex level and for proteins at the quaternary structure level, a level reached only by very small steps using yeast two hybrid system, far Western blotting, FRET and co-immunoprecipitation. This opens new directions of research including: 1) the identification of transient protein-protein interactions from signal transduction pathways (transductome) and its modifications (e.g. phospho-transductome) 2) protein complexes containing one protein during development (developmental protein-protein interactomics), 3) identification and characterization of protein complexes at the subunit level and post-translational modification level from both normal and diseased organelles. Finally, application of BN-PAGE in analyzing protein-protein interactions to investigate both normal and diseased extracellular fluids (e.g. serum/plasma, spinal fluid, tears or saliva) opens immense research opportunities for understanding a disease at the protein complex level.

Abbreviations

BN-PAGE, Blue Native PAGE; CN-PAGE, Colorless Native PAGE; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mw, molecular weight; WB, Western blotting; MS, mass spectrometry, LC-MS/MS, liquid chromatography mass spectrometry, MALDI-MS, Matrix Assisted Laser Desorption Ionization Mass Spectrometry; m/z, mass/charge; CID, collision-induced dissociation.

Acknowledgments

The authors would like to thank Laura Mulderig, Todd Boyer, Scott Nichols, Nathan Vanderlan, Tim Mascal, Keith Compson, Michael Balloch, Catalin Doneanu, David Chamberlin, Cathy Bolland, Joanne Boland, Janet Gallager, Sean McCarthy, Cristian Cojocariu and Charles Pragnell from Waters Corporation for the collaborative discount that allowed us to purchase a Micromass Quadrupole Time of Flight (QTOF) Micro mass spectrometer. The authors would also like to thank Thomas A. Neubert from the Skirball Institute, New York University for donation of the TofSpec2E MALDI-TOF mass spectrometer and Steven Blais (Skirball Institute, New York University) and Jason Watson, Mark Welsh and Tom Adams (Waters Corporation) for technical support and advice in setting up the MALDI MS. The authors also thank Drs. Thomas A. Neubert, Paul M. Wassarman, Eveline Litscher, Wolfgang Haehnel, Vlad Artenie and Kenneth R. Woods for advice and support. This work was partially supported by Clarkson University (start-up to C.C.D.) and U.S. Army Research Office (DURIP program) through grant #W911NF-11-1-0304.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

References

1. Schagger, H. Native electrophoresis for isolation of mitochondrial oxidative phosphorylation protein complexes. *Methods Enzymol.* **1995**, *260*, 190–202.
2. Schagger, H.; Cramer, W. A.; von Jagow, G. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **1994**, *217*, 220–230.
3. Schagger, H.; von Jagow, G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **1991**, *199*, 223–231.
4. Darie, C. C.; Biniossek, M. L.; Winter, V.; Mutschler, B.; Haehnel, W. Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*. *FEBS J.* **2005**, *272*, 2705–2716.
5. Eubel, H.; Braun, H. P.; Millar, A. H. Blue-native PAGE in plants: A tool in analysis of protein-protein interactions. *Plant Methods* **2005**, *1*, 11.
6. van Lis, R.; Atteia, A.; Mendoza-Hernandez, G.; Gonzalez-Halphen, D. Identification of novel mitochondrial protein components of *Chlamydomonas reinhardtii*. A proteomic approach. *Plant Physiol.* **2003**, *132*, 318–330.
7. Ploscher, M.; Granvogl, B.; Zoryan, M.; Reisinger, V.; Eichacker, L. A. Mass spectrometric characterization of membrane integral low molecular weight proteins from photosystem II in barley etioplasts. *Proteomics* **2009**, *9*, 625–635.
8. Heinemeyer, J.; Eubel, H.; Wehmhoner, D.; Jansch, L.; Braun, H. P. Proteomic approach to characterize the supramolecular organization of photosystems in higher plants. *Phytochemistry* **2004**, *65*, 1683–1692.
9. Silvestri, E.; Cioffi, F.; Glinni, D.; Ceccarelli, M.; Lombardi, A.; de Lange, P.; Chambery, A.; Severino, V.; Lanni, A.; Goglia, F.; Moreno, M. Pathways affected by 3,5-diiodo-l-thyronine in liver of high fat-fed rats: evidence from two-dimensional electrophoresis, blue-native PAGE, and mass spectrometry. *Mol. Biosyst.* **2010**, *6*, 2256–2271.
10. Andringa, K. K.; Bailey, S. M. Detection of protein thiols in mitochondrial oxidative phosphorylation complexes and associated proteins. *Methods Enzymol.* **2010**, *474*, 83–108.
11. Andringa, K. K.; King, A. L.; Eccleston, H. B.; Mantena, S. K.; Landar, A.; Jhala, N. C.; Dickinson, D. A.; Squadrito, G. L.; Bailey, S. M. Analysis of the liver mitochondrial proteome in response to ethanol and S-adenosylmethionine treatments: Novel molecular targets of disease and hepatoprotection. *Am. J. Physiol.: Gastrointest Liver Physiol.* **2010**, *298*, G732–745.
12. Reisinger, V.; Eichacker, L. A. Organelle proteomics: reduction of sample complexity by enzymatic in-gel selection of native proteins. *Methods Mol. Biol.* **2009**, *564*, 325–333.
13. Klepsch, M.; Schlegel, S.; Wickstrom, D.; Friso, G.; van Wijk, K. J.; Persson, J. O.; de Gier, J. W.; Wagner, S. Immobilization of the first dimension in 2D blue native/SDS-PAGE allows the relative quantification of membrane proteomes. *Methods* **2008**, *46*, 48–53.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

14. Reisinger, V.; Eichacker, L. A. Solubilization of membrane protein complexes for blue native PAGE. *J. Proteomics* **2008**, *71*, 277–283.
15. Bailey, S. M.; Andringa, K. K.; Landar, A.; Darley-Usmar, V. M. Proteomic approaches to identify and characterize alterations to the mitochondrial proteome in alcoholic liver disease. *Methods Mol. Biol.* **2008**, *447*, 369–380.
16. Reisinger, V.; Eichacker, L. A. How to analyze protein complexes by 2D blue native SDS-PAGE. *Proteomics* **2007**, *7* (Supplement 1), 6–16.
17. Aivaliotis, M.; Karas, M.; Tsiotis, G. An alternative strategy for the membrane proteome analysis of the green sulfur bacterium *Chlorobium tepidum* using blue native PAGE and 2-D PAGE on purified membranes. *J. Proteome Res.* **2007**, *6*, 1048–1058.
18. Babusiak, M.; Man, P.; Petrak, J.; Vyoral, D. Native proteomic analysis of protein complexes in murine intestinal brush border membranes. *Proteomics* **2007**, *7*, 121–129.
19. Aivaliotis, M.; Karas, M.; Tsiotis, G. High throughput two-dimensional blue-native electrophoresis: A tool for functional proteomics of cytoplasmatic protein complexes from *Chlorobium tepidum*. *Photosynth. Res.* **2006**, *88*, 143–157.
20. Di Pancrazio, F.; Bisetto, E.; Alverdi, V.; Mavelli, I.; Esposito, G.; Lippe, G. Differential steady-state tyrosine phosphorylation of two oligomeric forms of mitochondrial F0F1ATPsynthase: A structural proteomic analysis. *Proteomics* **2006**, *6*, 921–926.
21. Camacho-Carvajal, M. M.; Wollscheid, B.; Aebersold, R.; Steimle, V.; Schamel, W. W. Two-dimensional Blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: A proteomics approach. *Mol. Cell. Proteomics* **2004**, *3*, 176–182.
22. McDonald, T. G.; Van Eyk, J. E. Mitochondrial proteomics. Undercover in the lipid bilayer. *Basic Res. Cardiol.* **2003**, *98*, 219–227.
23. Darie, C. C.; Janssen, W. G.; Litscher, E. S.; Wassarman, P. M. Purified trout egg vitelline envelope proteins VEbeta and VEgamma polymerize into homomeric fibrils from dimers in vitro. *Biochim. Biophys. Acta* **2008**, *1784*, 385–392.
24. Litscher, E. S.; Janssen, W. G.; Darie, C. C.; Wassarman, P. M. Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions. *J. Cell. Physiol.* **2008**, *214*, 153–157.
25. Darie, C. C.; Shetty ,V.; Spellman D. S.; Zhang G.; Xu C.; Cardasis H. L.; Blais S.; Fenyo D.; Neubert, T. A. *Blue Native PAGE and Mass Spectrometry Analysis of the Ephrin Stimulation- Dependent Protein-Protein Interactions in NG108-EphB2 Cells*; Springer-Verlag: Düsseldorf, Germany, 2008.
26. Darie, C. C.; Deinhardt, K.; Zhang, G.; Cardasis, H. S.; Chao, M. V.; Neubert, T. A. Identifying transient protein-protein interactions in EphB2 signaling by Blue Native PAGE and mass spectrometry. *Proteomics* **2011**.
27. Reifschneider, N. H.; Goto, S.; Nakamoto, H.; Takahashi, R.; Sugawa, M.; Dencher, N. A.; Krause, F. Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D blue-native/SDS-PAGE. *J. Proteome Res.* **2006**, *5*, 1117–1132.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

28. Woods, A. G.; Guthrie, K. M.; Kurlawalla, M. A.; Gall, C. M. Deafferentation-induced increases in hippocampal insulin-like growth factor-1 messenger RNA expression are severely attenuated in middle aged and aged rats. *Neuroscience* **1998**, *83*, 663–668.
29. Woods, A. G.; Poulsen, F. R.; Gall, C. M. Dexamethasone selectively suppresses microglial trophic responses to hippocampal deafferentation. *Neuroscience* **1999**, *91*, 1277–1289.
30. Pitulescu, M. E.; Adams, R. H. Eph/ephrin molecules--A hub for signaling and endocytosis. *Genes Dev.* **2010**, *24*, 2480–2492.
31. Holland, S. J.; Gale, N. W.; Gish, G. D.; Roth, R. A.; Songyang, Z.; Cantley, L. C.; Henkemeyer, M.; Yancopoulos, G. D.; Pawson, T. Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J.* **1997**, *16*, 3877–3888.
32. Holland, S. J.; Gale, N. W.; Mbamalu, G.; Yancopoulos, G. D.; Henkemeyer, M.; Pawson, T. Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* **1996**, *383*, 722–725.
33. Holland, S. J.; Peles, E.; Pawson, T.; Schlessinger, J. Cell-contact-dependent signalling in axon growth and guidance: Eph receptor tyrosine kinases and receptor protein tyrosine phosphatase beta. *Curr. Opin. Neurobiol.* **1998**, *8*, 117–127.
34. Wilkinson, D. G. Multiple roles of EPH receptors and ephrins in neural development. *Nat. Rev. Neurosci.* **2001**, *2*, 155–164.
35. Dalva, M. B.; Takasu, M. A.; Lin, M. Z.; Shamah, S. M.; Hu, L.; Gale, N. W.; Greenberg, M. E. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell* **2000**, *103*, 945–956.
36. Robinson, V.; Smith, A.; Flenniken, A. M.; Wilkinson, D. G. Roles of Eph receptors and ephrins in neural crest pathfinding. *Cell Tissue Res.* **1997**, *290*, 265–274.
37. Adams, R. H. Molecular control of arterial-venous blood vessel identity. *J. Anat.* **2003**, *202*, 105–112.
38. Cheng, N.; Brantley, D. M.; Chen, J. The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev.* **2002**, *13*, 75–85.
39. Yancopoulos, G. D.; Klagsbrun, M.; Folkman, J. Vasculogenesis, angiogenesis, and growth factors: Ephrins enter the fray at the border. *Cell* **1998**, *93*, 661–664.
40. Himanen, J. P.; Nikolov, D. B. Eph receptors and ephrins. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 130–134.
41. Gale, N. W.; Holland, S. J.; Valenzuela, D. M.; Flenniken, A.; Pan, L.; Ryan, T. E.; Henkemeyer, M.; Strebhardt, K.; Hirai, H.; Wilkinson, D. G.; Pawson, T.; Davis, S.; Yancopoulos, G. D. Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **1996**, *17*, 9–19.
42. Carter, N.; Nakamoto, T.; Hirai, H.; Hunter, T. EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). *Nat. Cell Biol.* **2002**, *4*, 565–573.

43. Miao, H.; Burnett, E.; Kinch, M.; Simon, E.; Wang, B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat. Cell Biol.* **2000**, *2*, 62–69.
44. Vearing, C. J.; Lackmann, M. Eph receptor signalling; dimerisation just isn't enough. *Growth Factors* **2005**, *23*, 67–76.
45. Noren, N. K.; Pasquale, E. B. Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. *Cell Signal* **2004**, *16*, 655–666.
46. Ellis, C.; Kasmi, F.; Ganju, P.; Walls, E.; Panayotou, G.; Reith, A. D. A juxtamembrane autophosphorylation site in the Eph family receptor tyrosine kinase, Sek, mediates high affinity interaction with p59fyn. *Oncogene* **1996**, *12*, 1727–1736.
47. Pandey, A.; Andersen, J. S.; Mann, M. Use of mass spectrometry to study signaling pathways. *Sci. STKE* **2000**, pl1.
48. Pandey, A.; Duan, H.; Dixit, V. M. Characterization of a novel Src-like adapter protein that associates with the Eck receptor tyrosine kinase. *J. Biol. Chem.* **1995**, *270*, 19201–19204.
49. Pandey, A.; Lazar, D. F.; Saltiel, A. R.; Dixit, V. M. Activation of the Eck receptor protein tyrosine kinase stimulates phosphatidylinositol 3-kinase activity. *J. Biol. Chem.* **1994**, *269*, 30154–30157.
50. Pandey, A.; Podtelejnikov, A. V.; Blagoev, B.; Bustelo, X. R.; Mann, M.; Lodish, H. F. Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 179–184.
51. Stein, E.; Cerretti, D. P.; Daniel, T. O. Ligand activation of ELK receptor tyrosine kinase promotes its association with Grb10 and Grb2 in vascular endothelial cells. *J. Biol. Chem.* **1996**, *271*, 23588–23593.
52. Stein, E.; Huynh-Do, U.; Lane, A. A.; Cerretti, D. P.; Daniel, T. O. Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. *J. Biol. Chem.* **1998**, *273*, 1303–1308.
53. Stein, E.; Lane, A. A.; Cerretti, D. P.; Schoecklmann, H. O.; Schroff, A. D.; Van Etten, R. L.; Daniel, T. O. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev.* **1998**, *12*, 667–678.
54. Zhang, G.; Fenyo, D.; Neubert, T. A. Screening for EphB signaling effectors using SILAC with a linear ion trap-orbitrap mass spectrometer. *J. Proteome Res.* **2008**, *7*, 4715–4726.
55. Zhang, G.; Spellman, D. S.; Skolnik, E. Y.; Neubert, T. A. Quantitative phosphotyrosine proteomics of EphB2 signaling by stable isotope labeling with amino acids in cell culture (SILAC). *J. Proteome Res.* **2006**, *5*, 581–588.
56. DeLaBarre, B.; Brunger, A. T. Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat. Struct. Biol.* **2003**, *10*, 856–863.
57. Hatzivassiliou, G.; Zhao, F.; Bauer, D. E.; Andreadis, C.; Shaw, A. N.; Dhanak, D.; Hingorani, S. R.; Tuveson, D. A.; Thompson, C. B. ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* **2005**, *8*, 311–321.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

58. Bauer, D. E.; Hatzivassiliou, G.; Zhao, F.; Andreadis, C.; Thompson, C. B. ATP citrate lyase is an important component of cell growth and transformation. *Oncogene* **2005**, *24*, 6314–6322.
59. McNaught, K. S.; Olanow, C. W.; Halliwell, B.; Isacson, O.; Jenner, P. Failure of the ubiquitin-proteasome system in Parkinson's disease. *Nat. Rev. Neurosci.* **2001**, *2*, 589–594.
60. Brown, M. G.; Monaco, J. J. Biochemical purification of distinct proteasome subsets. *Enzyme Protein* **1993**, *47*, 343–353.
61. Hendil, K. B.; Welinder, K. G.; Pedersen, D.; Uerkvitz, W.; Kristensen, P. Subunit stoichiometry of human proteasomes. *Enzyme Protein* **1993**, *47*, 232–240.
62. Seelig, A.; Boes, B.; Kloetzel, P. M. Characterization of mouse proteasome subunit MC3 and identification of proteasome subtypes with different cleavage characteristics. Proteasome subunits, proteasome subpopulations. *Enzyme Protein* **1993**, *47*, 330–342.
63. Hendil, K. B.; Kristensen, P.; Uerkvitz, W. Human proteasomes analysed with monoclonal antibodies. *Biochem. J.* **1995**, *305* (Part 1), 245–252.
64. Akaishi, T.; Shiomi, T.; Sawada, H.; Yokosawa, H. Purification and properties of the 26S proteasome from the rat brain: Evidence for its degradation of myelin basic protein in a ubiquitin-dependent manner. *Brain Res.* **1996**, *722*, 139–144.
65. Henry, L.; Baz, A.; Chateau, M. T.; Caravano, R.; Scherrer, K.; Bureau, J. P. Proteasome (prosome) subunit variations during the differentiation of myeloid U937 cells. *Anal. Cell. Pathol.* **1997**, *15*, 131–144.
66. Seeger, M.; Ferrell, K.; Dubiel, W. The 26S proteasome: A dynamic structure. *Mol. Biol. Rep.* **1997**, *24*, 83–88.
67. Wang, X.; Chen, C. F.; Baker, P. R.; Chen, P. L.; Kaiser, P.; Huang, L. Mass spectrometric characterization of the affinity-purified human 26S proteasome complex. *Biochemistry* **2007**, *46*, 3553–3565.
68. Yu, Y.; Ji, H.; Doudna, J. A.; Leary, J. A. Mass spectrometric analysis of the human 40S ribosomal subunit: Native and HCV IRES-bound complexes. *Protein Sci.* **2005**, *14*, 1438–1446.
69. Damoc, E.; Fraser, C. S.; Zhou, M.; Videler, H.; Mayeur, G. L.; Hershey, J. W.; Doudna, J. A.; Robinson, C. V.; Leary, J. A. Structural characterization of the human eukaryotic initiation factor 3 protein complex by mass spectrometry. *Mol. Cell. Proteomics* **2007**, *6*, 1135–1146.
70. Fogli, A.; Malinverni, C.; Thadikkaran, L.; Combes, P.; Perret, F.; Crettaz, D.; Tissot, J. D.; Boespflug-Tanguy, O.; Stocklin, R.; Bulet, P. Peptidomics and proteomics studies of transformed lymphocytes from patients mutated for the eukaryotic initiation factor 2B. *J. Chromatogr., B* **2006**, *840*, 20–28.
71. Sanders, S. L.; Jennings, J.; Canutescu, A.; Link, A. J.; Weil, P. A. Proteomics of the eukaryotic transcription machinery: Identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. *Mol. Cell. Biol.* **2002**, *22*, 4723–4738.
72. Zhou, M.; Sandercock, A. M.; Fraser, C. S.; Ridlova, G.; Stephens, E.; Schenauer, M. R.; Yokoi-Fong, T.; Barsky, D.; Leary, J. A.; Hershey, J. W.;

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Doudna, J. A.; Robinson, C. V. Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 18139–18144.

73. Lu, L.; Komada, M.; Kitamura, N. Human Hrs, a tyrosine kinase substrate in growth factor-stimulated cells: cDNA cloning and mapping of the gene to chromosome 17. *Gene* **1998**, *213*, 125–132.
74. Kobayashi, H.; Tanaka, N.; Asao, H.; Miura, S.; Kyuuma, M.; Semura, K.; Ishii, N.; Sugamura, K. Hrs, a mammalian master molecule in vesicular transport and protein sorting, suppresses the degradation of ESCRT proteins signal transducing adaptor molecule 1 and 2. *J. Biol. Chem.* **2005**, *280*, 10468–10477.
75. Spellman, D. S.; Deinhardt, K.; Darie, C. C.; Chao, M. V.; Neubert, T. A. Stable isotopic labeling by amino acids in cultured primary neurons: Application to brain-derived neurotrophic factor-dependent phosphotyrosine-associated signaling. *Mol. Cell. Proteomics* **2008**, *7*, 1067–1076.
76. Darie, C. C.; Biniossek, M. L.; Gawinowicz, M. A.; Milgrom, Y.; Thumfart, J. O.; Jovine, L.; Litscher, E. S.; Wassarman, P. M. Mass spectrometric evidence that proteolytic processing of rainbow trout egg vitelline envelope proteins takes place on the egg. *J. Biol. Chem.* **2005**, *280*, 37585–37598.
77. Darie, C. C.; Biniossek, M. L.; Jovine, L.; Litscher, E. S.; Wassarman, P. M. Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. *Biochemistry* **2004**, *43*, 7459–7478.
78. Jovine, L.; Darie, C. C.; Litscher, E. S.; Wassarman, P. M. *Zona pellucida* domain proteins. *Annu. Rev. Biochem.* **2005**, *74*, 83–114.
79. Jovine, L.; Janssen, W. G.; Litscher, E. S.; Wassarman, P. M. The PLAC1-homology region of the ZP domain is sufficient for protein polymerisation. *BMC Biochem.* **2006**, *7*, 11.
80. Jovine, L.; Litscher, E.; Wassarman, P. M. Egg zona pellucida, egg vitelline envelope, and related extracellular glycoproteins. In *Advances in Developmental Biology and Biochemistry*; DePamphilis, M. L., Ed.; Elsevier: Amsterdam, 2002; pp 31−54.
81. Jovine, L.; Qi, H.; Williams, Z.; Litscher, E.; Wassarman, P. M. The ZP domain is a conserved module for polymerization of extracellular proteins. *Nat. Cell Biol.* **2002**, *4*, 457–461.
82. Jovine, L.; Qi, H.; Williams, Z.; Litscher, E. S.; Wassarman, P. M. A duplicated motif controls assembly of zona pellucida domain proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 5922–5927.
83. Darie, C. C.; De Pascalis, L.; Mutschler, B.; Haehnel, W. Studies of the Ndh complex and photosystem II from mesophyll and bundle sheath chloroplasts of the C4-type plant *Zea mays*. *J. Plant Physiol.* **2006**, *163*, 800–808.
84. Prommeenate, P.; Lennon, A. M.; Markert, C.; Hippler, M.; Nixon, P. J. Subunit composition of NDH-1 complexes of Synechocystis sp. PCC 6803: Identification of two new ndh gene products with nuclear-encoded homologues in the chloroplast Ndh complex. *J. Biol. Chem.* **2004**, *279*, 28165–28173.

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

85. Joet, T.; Cournac, L.; Peltier, G.; Havaux, M. Cyclic electron flow around photosystem I in C(3) plants. In vivo control by the redox state of chloroplasts and involvement of the NADH-dehydrogenase complex. *Plant Physiol.* **2002**, *128*, 760–769.
86. Casano, L. M.; Zapata, J. M.; Martin, M.; Sabater, B. Chlororespiration and poising of cyclic electron transport. Plastoquinone as electron transporter between thylakoid NADH dehydrogenase and peroxidase. *J. Biol. Chem.* **2000**, *275*, 942–948.
87. Sazanov, L. A.; Burrows, P. A.; Nixon, P. J. The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. *FEBS Lett* **1998**, *429*, 115–118.
88. Catala, R.; Sabater, B.; Guera, A. Expression of the plastid ndhF gene product in photosynthetic and non-photosynthetic tissues of developing barley seedlings. *Plant Cell Physiol.* **1997**, *38*, 1382–1388.
89. Burrows, P. A.; Sazanov, L. A.; Svab, Z.; Maliga, P.; Nixon, P. J. Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndh genes. *EMBO J.* **1998**, *17*, 868–876.
90. Sazanov, L. A.; Burrows, P. A.; Nixon, P. J. The plastid ndh genes code for an NADH-specific dehydrogenase: Isolation of a complex I analogue from pea thylakoid membranes. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 1319–1324.
91. Guedeney, G.; Corneille, S.; Cuine, S.; Peltier, G. Evidence for an association of ndh B, ndh J gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex. *FEBS Lett.* **1996**, *378*, 277–280.
92. Ladig, R.; Sommer, M. S.; Hahn, A.; Leisegang, M. S.; Papasotiriou, D. G.; Ibrahim, M.; Elkehal, R.; Karas, M.; Zickermann, V.; Gutensohn, M.; Brandt, U.; Klosgen, R. B.; Schleiff, E. A high-definition native polyacrylamide gel electrophoresis system for the analysis of membrane complexes. *Plant J.* **2011**.
93. Peng, Y.; Luo, Y.; Yu, T.; Xu, X.; Fan, K.; Zhao, Y.; Yang, K. A blue native-PAGE analysis of membrane protein complexes in *Clostridium thermocellum*. *BMC Microbiol.* **2011**, *11*, 22.
94. Wessels, H. J.; Vogel, R. O.; van den Heuvel, L.; Smeitink, J. A.; Rodenburg, R. J.; Nijtmans, L. G.; Farhoud, M. H. LC-MS/MS as an alternative for SDS-PAGE in blue native analysis of protein complexes. *Proteomics* **2009**, *9*, 4221–4228.
95. Meng, B.; Qian, Z.; Wei, F.; Wang, W.; Zhou, C.; Wang, Z.; Wang, Q.; Tong, W.; Ma, Y.; Xu, N.; Liu, S. Proteomic analysis on the temperature-dependent complexes in Thermoanaerobacter tengcongensis. *Proteomics* **2009**, *9*, 3189–3200.
96. Sanders, P. R.; Cantin, G. T.; Greenbaum, D. C.; Gilson, P. R.; Nebl, T.; Moritz, R. L.; Yates, J. R., 3rd; Hodder, A. N.; Crabb, B. S. Identification of protein complexes in detergent-resistant membranes of *Plasmodium falciparum* schizonts. *Mol. Biochem. Parasitol.* **2007**, *154*, 148–157.
97. Pyndiah, S.; Lasserre, J. P.; Menard, A.; Claverol, S.; Prouzet-Mauleon, V.; Megraud, F.; Zerbib, F.; Bonneu, M. Two-dimensional blue native/SDS gel

Downloaded by PENNSYLVANIA STATE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch012

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Chapter 13

Mass Spectrometry for Proteomics-Based Investigation of Oxidative Stress and Heat Shock Proteins

Izabela Sokolowska,[1] Alisa G. Woods,[1] Jessica Wagner,[1] Jeannette Dorler,[1] Kelly Wormwood,[1] Johannes Thome,[2] and Costel C. Darie*,[1]

**[1]Department of Chemistry & Biomolecular Science,
Biochemistry & Proteomics Group, Clarkson University,
8 Clarkson Avenue, Potsdam, New York, 13699-5810, U.S.A.
[2]Department of Psychiatry, University of Rostock,
Gehlsheimer Straße 20, D-18147 Rostock, Germany
*Tel: (315) 268-7763. Fax: (315) 268-6610. E-mail: cdarie@clarkson.edu.**

Proteomic analysis using mass spectrometry (MS) is the study of proteins, ranging from the supracellular and subcellular levels to the intracellular and extracellular levels. Proteomic analysis can also occur at the peptide, protein or protein complex level. Oxidative stress refers to the generation of reactive oxygen species (ROS), which have the potential to cause cellular and tissue damage. Oxidative stress triggered by ROS causes various changes in a cell's proteome. One of many responses to oxidative stress is increased expression of Heat Shock Proteins (HSPs). Oxidative stress can be investigated at the level of the peptide, protein, protein complex or proteome using MS. Proteomic analysis of oxidative stress may be difficult at both the protein identification and protein quantitation levels. One of the biggest challenges is the number of amino acids that are modified by simple oxidation and the proteomic consequences of these oxidations. Here, we describe the principle of mass spectrometry and proteomics, oxidative stress and its effects on the modification of amino acids. We also describe HSPs and their roles in oxidative stress, with particular investigation of two members of the HSP70 family (GRP78 and HSP70). In

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

addition, we describe a new function of members of the HSP70 family as potential receptors for a recently discovered pituitary factor. Finally, in our concluding remarks we assess the role of mass spectrometry and proteomics in the investigation of oxidative stress.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Introduction

Mass spectrometry (MS) is a crucial proteomic method that every academic center should use (*1–8*). MS can be used to analyze a variety of chemical and biological compounds. These compounds include products of chemical synthesis or degradation, biological building blocks and their products, (including proteins, nucleic acids, lipids, and glycans). Within the MS field different specialized subfields have emerged, depending on the chemical or biological entities that are being analyzed. Among these subfields, there is MS of small molecules, MS of large molecules, or biological MS, where the molecules analyzed are biomolecules. Depending on the type of biological entities that are investigated by mass spectrometry, there are different fields, including: proteomics, lipidomics, glycomics and others. Proteomics analyzes proteins, peptides, aglycans, protein-protein interactions, or post-translational modifications (PTMs).

The workflow in a proteomics experiment involves sample fractionation by 1, 2, or more biochemical approaches, followed by enzymatic digestion (usually trypsin), peptide extraction, and MS analysis (*1*). Data analysis leads to identification of one or more proteins. Other potential aspects for analysis include post-translational modifications and interaction partners (protein-protein interactions) (*9–17*). A schematic of a proteomics experiment is shown in Figure 1A.

Proteomic analysis can be accomplished at several levels including: supracellular, subcellular, intracellular, extracellular, as well as the peptide, protein or protein complex level. Proteomics also may be classical, functional or focus on a particular sub-proteome such as phosphoproteomics or glycoproteomics. Based on the type of protein samples (proteomes or sub-proteomes) that are analyzed, proteomics may be classified as schematically shown in Figure 1B. Proteomic analysis can be qualitative for protein identification and quantitative for the determination of protein amounts. These analyses are usually performed using a mass spectrometer, composed of an ionization source, a mass analyzer and a detector. A schematic of a mass spectrometer is shown in Figure 1C. The mass spectrometers that are used in proteomics have primarily two types of ionization sources: Matrix Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization. Depending on the ionization sources, the mass spectrometers (and the techniques) used are MALDI-MS and ESI-MS. In this chapter, we will describe how proteins and peptides are analyzed by MS, and the type of information regarding oxidative stress that can be extracted. Here, we will refer to the compounds that are being analyzed as peptides, but the methods *per se* can be applied to almost any compound.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

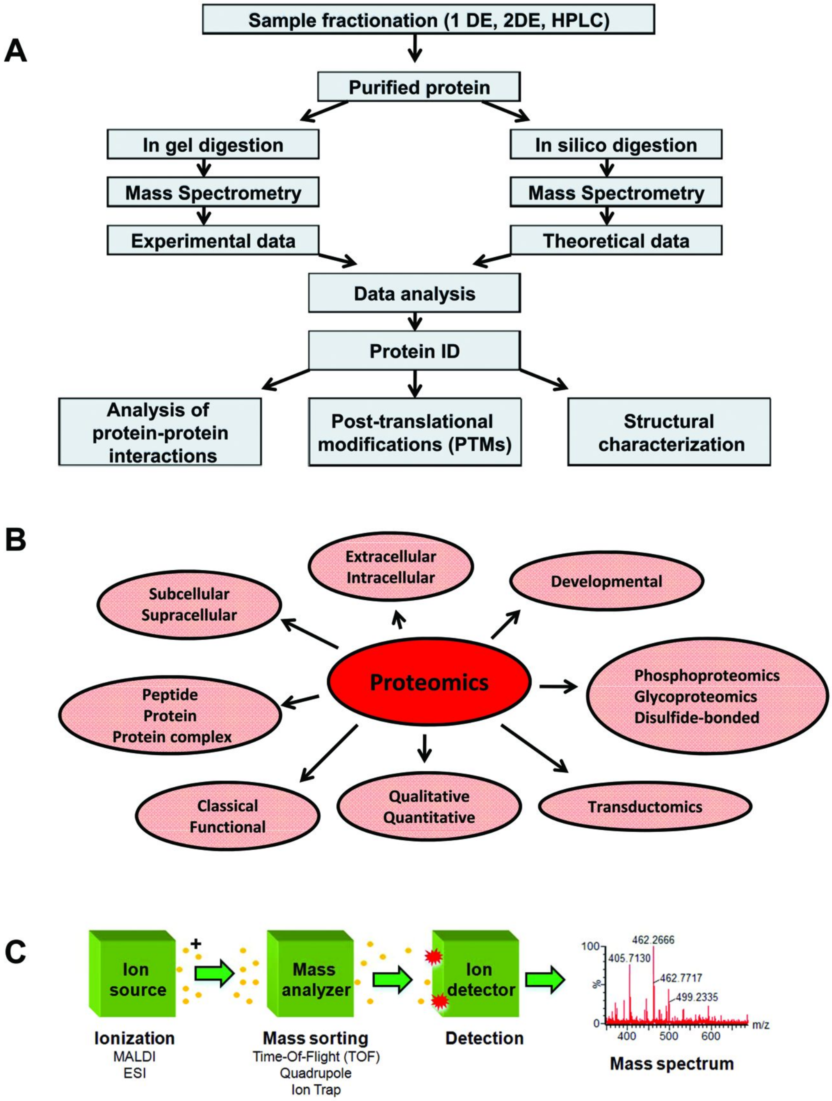

A
Sample fractionation (1 DE, 2DE, HPLC)
Purified protein
In gel digestion
In silico digestion
Mass Spectrometry
Mass Spectrometry
Experimental data
Theoretical data
Data analysis
Protein ID
Analysis of protein-protein interactions
Post-translational modifications (PTMs)
Structural characterization
B
Proteomics
Extracellular
Intracellular
Subcellular
Supracellular
Developmental
Phosphoproteomics
Glycoproteomics
Disulfide-bonded
Peptide
Protein
Protein complex
Classical
Functional
Qualitative
Quantitative
Transductomics
C
Ion source
+
Mass analyzer
Ion detector
Ionization
MALDI
ESI
Mass sorting
Time-Of-Flight (TOF)
Quadrupole
Ion Trap
Detection
100
462.2666
405.7130
462.7717
%
499.2335
0
m/z
400
500
600
Mass spectrum

*Figure 1. The schematic workflow in a proteomics experiment **(A)**. Schematic classification of proteomics and its applications **(B)**. Schematic of a mass spectrometer **(C)**.*

Biochemical Fractionation

Biochemical fractionation is an approach by which various proteins are separated from each other using their physico-chemical properties, according to the final goal of the experiment that needs to be achieved. Biochemical fractionation is the first of three steps in a proteomics experiment. Biochemical fractionation is perhaps the cornerstone of a proteomics experiment. Without fractionation, one may still perform a proteomics experiment (and therefore it is not always required); however, the outcome and the usefulness of that experiment may be diminished without fractionation. Fractionation takes advantages of the physico-chemical properties of proteins (or compounds of interest) such as molecular mass, isoelectric point, ionic species at neutral pH, etc). Examples of biochemical fractionations include chromatography (affinity chromatography, ion exchange chromatography, size-exclusion chromatography), centrifugation and ultracentrifugation, electrophoresis, precipitation, etc. In the simplest case scenario, the protein sample is separated by electrophoresis, usually sodium dodecyl-sulfate polyacrylamide electrophoresis (SDS-PAGE). The advantage of this method is that proteins are reduced and denatured and then separated according to their molecular mass. Reduction of proteins takes place through disruption of intramolecular and intermolecular cysteine-based disulfide bridges, which allows the protein to become more linear and in a monomeric form, while denaturation of these proteins with a strong detergent (e.g. SDS) will completely linearize the protein and allow researchers to determine the molecular mass of that protein using SDS-PAGE. Variants of electrophoresis are also used, depending on the final goal of the research project. An example of a variant of electrophoresis is Tricine-PAGE (*18*, *19*). Tricine-PAGE uses the same principle of separation as SDS-PAGE, but its separating resolution is increased for low molecular weight proteins and peptides (2-20 kDa). Another example of a variant of electrophoresis is native electrophoresis and its variants such as Blue Native PAGE (BN-PAGE), colorless native PAGE (CN-PAGE) and detergent-less SDS-PAGE (native PAGE) (*1*, *4*, *6*, *9–13*, *20–22*). BN-PAGE separates protein complexes according to their molecular weight, due to the external charge induced by Coomassie dye. When the dye is omitted, the separation of protein complexes still takes place, but only according to their internal charge and independent of their mass. There are many other separation and fractionation methods such as chromatography, chromatofocusing, centrifugation and ultracentrifugation, as well as hyphenated techniques, during which one may combine two or more separation or fractionation methods. For example, in proteomics, a long-used hyphenated fractionation technique is two dimensional electrophoresis, which includes separation of proteins by isoelectric focusing followed by SDS-PAGE (*3*, *7*, *23–33*). This method, due to its high resolution, is still in use and its variant; differential gel electrophoresis (DIGE) can be combined with MS to constitute a powerful gel-based proteomics approach. Another hyphenated technique increasingly being used is the combination of BN-PAGE and SDS-PAGE, followed by MS, which allows researchers to analyze stable and transient protein-protein interactions, protein complexes and their subunit composition and post-translational modifications. Apart from fractionation via PAGE technologies,

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

alternative methods such as pre-coated chips, centrifugal filters and magnetic beads, have been developed and successfully used (*34*, *35*).

The success of a proteomics experiment using biochemical fractionation, may be different, depending on the final goal of the experiment. For example, assuming that one uses human sera for a proteomics experiment (e.g. 10,000 proteins), any proteomics experiment using unfractionated human sera will be successful. One will always identify proteins; the most abundant proteins (e.g. top 100-1000 proteins). However, to identify the low-abundant proteins, one must remove the highly-abundant ones in a process named depletion (e.g. affinity chromatography), which is a particular case of biochemical fractionation. Therefore, the proteomics experiment is successful only if the final goal of identifying the proteins of interest, with biological significance is achieved. Similarly, biochemical fractionation is performed only if it helps with the successful identification of proteins of physiological or pathological significance. Once biochemical fractionation is performed, the proteins of interest are further analyzed by mass spectrometry.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Mass Spectrometry

All mass spectrometers have three essential components: an ion source, a mass analyzer, and a detector. Ions are produced from the sample in the ion source using a specific ionization method, are separated in the mass analyzer based on their mass-to-charge (*m/z*) ratios and then detected, usually by an electron multiplier. The data system (which could be considered a fourth component) produces a mass spectrum, which is a plot of ion abundance versus *m/z*. Ionization takes place in the ion source. In order to be analyzed in the mass analyzer, a compound has to be ionized and then transferred from the liquid phase into the gaseous phase. Therefore, ionization of the compounds to be analyzed in the ion source is the first event that happens in a mass spectrometry-based experiment. Ionization of the compounds does not always work well and sometimes optimization procedures must be performed. Ionization of different compounds to be analyzed depends on the pH at which ionization is performed, on the electrical potential at the ion source and on the ion optics. At low pH, the compounds that can be protonated (e.g. amines or amino-containing compounds) are more abundant, while at high pH, the de-protonated compounds are more abundant. When the electrical potential at the ion source is positive, ionization is in positive ion mode and when the electrical potential at the ion source is negative, ionization is in negative ion mode. Therefore, there are two types of ionization: 1) positive ionization, in which compounds are analyzed mostly under acidic conditions, are protonated and the electrical potential at the ion source is positive and 2) negative ionization, in which compounds are analyzed under basic conditions, are de-protonated and the electrical potential at the ion source is negative. Here, we will focus only on positive ionization, because it is the most widely used ionization method for analyzing peptides and proteins. We will also refer only to MALDI and ESI as ionization methods. These ionization techniques (MALDI or ESI) produce primarily intact protonated molecular ions of peptides and proteins. MALDI and ESI are the two most widely used ionization methods for peptides and proteins and the mass spectrometers that have this ion source are named, as mentioned above, MALDI-MS and ESI-MS. Mass spectrometers may have various types of mass analyzers that separate ions using different principles. Among the most used analyzers are time of flight (TOF), ion trap (IT), and quadrupole (Q) mass analyzers. A simple mass spectrometer has one analyzer (Q or TOF), but more sophisticated mass spectrometers usually have a combination of two or three identical or different mass analyzers (e.g. Q-TOF, Q-Trap, QQQ). The MS Detectors are usually electron multipliers, photodiode arrays, microchannelplates or image current detectors.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

MALDI-TOF MS

MALDI-TOF MS or MALDI-MS is the method of choice for determination of the mass of a peptide or protein and for identification of a protein from a peptide mixture using peptide mass fingerprinting. In MALDI-MS, the peptide mixture is co-crystallized under acidic conditions with a matrix (UV-absorbing compounds, e.g. dihydrobenzoic acid, sinapinic acid, alpha-hydroxycinnamic acid) on a plate and a laser beam ionizes the matrix and peptide molecules, that start to fly under an electrical field. The laser is used to thermally desorb the matrix and the molecules. Peptides are ionized by proton transfer from the matrix molecules, fly through the time of flight (TOF) tube and are detected in the detector as a mass spectrum. In order to fly through the TOF tube and be detected, the peptides have to be accelerated and focused in the TOF analyzer. Once the peptides enter the mass spectrometer, they fly through the mass analyzer according to the formula: $[M + zH]/z$, where M is the mass of the peptide and z is the charge that the peptide carries; H is the mass of Hydrogen and is 1.007825035 atomic mass units. In proteomics, $[M + zH]/z$ or $[M + z]/z$ is abbreviated as m/z. In MALDI-MS, the charge z is mostly (+1) and the peptides are mostly observed as $[M+1 \times 1]/1$ or $[M+1]/1$. Therefore, the peptides are mostly ionized by one proton and are recorded in the mass spectrum as protonated $[MH]^+$ peaks. In the mass spectrum (assuming that the protein of interest was digested) one peak corresponds to one peptide (either from the protein of interest or from a contaminant protein such as keratin) and many peaks corresponding to peptides from the same protein will identify that protein in a database using peptide mass fingerprinting. Figure 2 shows the principle of MALDI-MS (Figure 2A) and an example of a MALDI-MS spectrum of a bovine serum albumin peptide mixture (Figure 2B). Peptide mass fingerprinting in the database spectrum search, leading to identification of this protein, is shown in Figure 2C.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

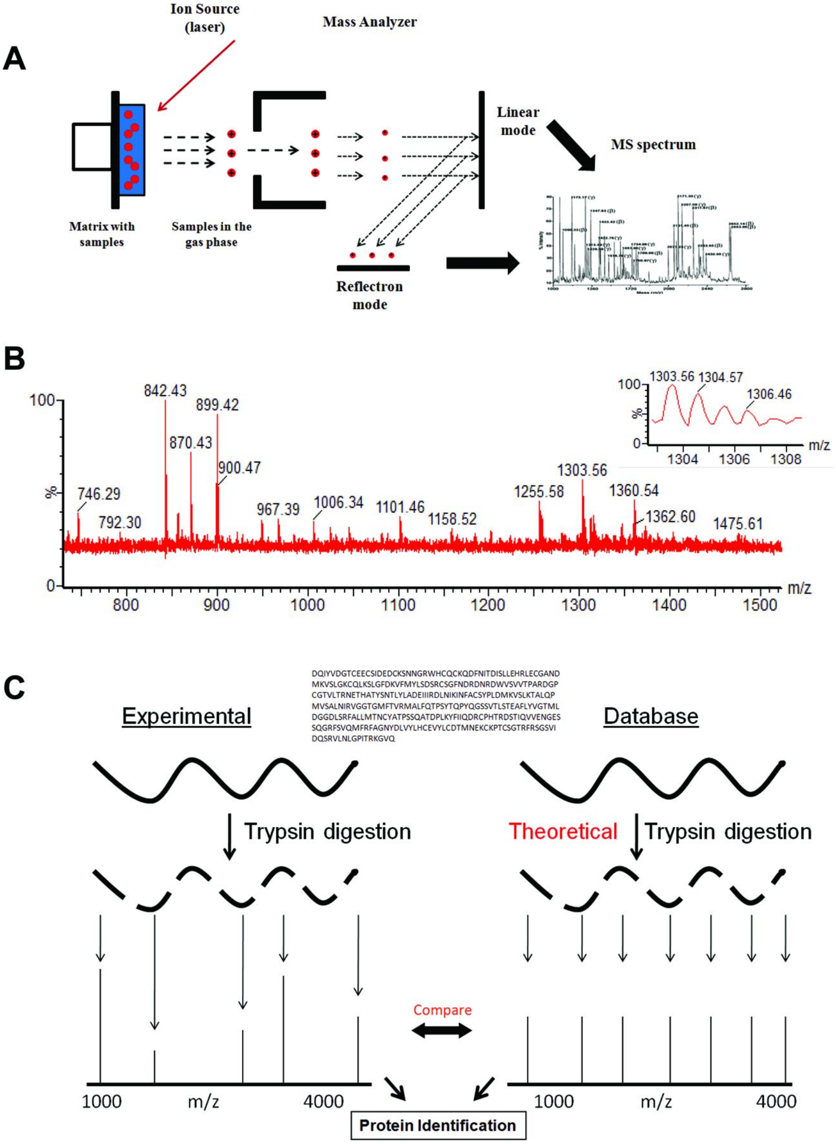

A
Ion Source
(laser)
Mass Analyzer
Linear mode
MS spectrum
Matrix with samples
Samples in the gas phase
Reflectron mode
B
100
%
0
746.29
792.30
842.43
870.43
899.42
900.47
967.39
1006.34
1101.46
1158.52
1255.58
1303.56
1360.54
1362.60
1475.61
800
900
1000
1100
1200
1300
1400
1500
m/z
1303.56
1304.57
1306.46
1304
1306
1308
C
Experimental
Database
DQIYVDGTCEECSIDEDCKSNNGRWHCQCKQDFNITDISLLEHRLECGAND
MKVSLGKCQLKSLGFDKVFMYLSDSRCSGFNDRDNRDWVSVVTPARDGP
CGTVLTRNETHATYSNTLYLADEIIIRDLNIKINFACSYPLDMKVSLKTALQP
MVSALNIRVGGTGMFTVRMALFQTPSYTQPYQGSSVTLSTEAFLYVGTML
DGGDLSRFALLMTNCYATPSSQATDPLKYFIIQDRCPHTRDSTIQVVENGES
SQGRFSVQMFRFAGNYDLVYLHCEVYLCDTMNEKCKPTCSGTRFRSGSVI
DQSRVLNLGPITRKGVQ
Trypsin digestion
Theoretical
Trypsin digestion
Compare
1000
m/z
4000
1000
m/z
4000
Protein Identification

*Figure 2. MALDI-TOF MS. **(A)** MALDI TOF mass spectrometer has an ion source, a mass analyzer (TOF linear or reflectron) and a detector, where the mass spectrum is detected and recorded. In linear mode (TOF linear, the ions travel a short time through the TOF tube and the resolution is lower. It is mainly used for proteins. In reflectron mode, ions travel a longer time through the TOF tube and the resolution is higher than in the linear mode. It is mainly used for peptides. **(B)** A MALDI MS spectrum consist of singly charged peaks; one such peak is enlarged to illustrate the charge state of the peak (single charged or +1). **(C)** Principle of protein identification using MALDI MS and peptide*

mass fingerprinting. In most MS-based proteomics experiments, the protein is cleaved into small pieces (peptides) by trypsin, the peptide mixture is analyzed by MALDI-MS and a spectrum is collected. During the database search, a similar experiment is performed in silico (a theoretical experiment in computer), but the cleavage is performed in all proteins from a database. Statistical analysis then tries to fit the experimental mass spectrum with one of the thousands of theoretical spectra. The best match between the theoretical and the experimental spectra then lead to identification of a protein.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

ESI-Q-TOF MS

In ESI-MS, unlike in MALDI-MS, in which peptides are ionized from the solid phase, peptides are ionized in the liquid phase, under high electric current according to the same [M + z]/z formula. Also, unlike in MALDI-MS, where peptides are mostly singly charged, in ESI-MS peptides are mostly double or multiply charged. The principle of ESI-MS is shown in Figure 3A. An example of an ESI-MS spectrum of double- and triple-charged peaks corresponding to the same peptide is shown in Figure 3B. When the peaks are double or triple charged, the peptide contains two or three protons. Since in the mass spectrometer the peptides fly according to mass-to-charge [M+z]/z, calculation of the molecular mass of the peptide is performed according to the same formula. Usually, for double- or triple-charged peaks, the first peak in the isotopic cluster is monoisotopic, meaning that the peaks contain only major isotopes (C12, H1, N14 and so on). Therefore, calculation of the molecular mass is performed for the monoisotopic peak. In addition to measuring the masses of the peptides, a mass spectrometer can also obtain sequence information for a particular peptide, due to the susceptibility of peptides to fragmentation (mostly at the peptidic bonds) into smaller pieces in a process called Collision-Induced Dissociation (CID; CID is not available in all mass spectrometers). In this process, the mass spectrometer records the spectra of the peptides (MS mode) and the peak of interest is selected and fragmented by CID (MS/MS mode) through collision with an inert gas (e.g. Argon gas). Collision usually takes place in a collision cell, although fragmentation of peptides may take place elsewhere, as well (e.g. in source fragmentation). Increase of the energy of the ion upon collision with the inert gas will make this ion unstable, a condition that will lead to fragmentation of the ions (and produce fragment ions). Mass spectral analysis of these fragment ions (product ions) into a second or third analyzer (not needed if an ion trap is used) will produce a spectrum named MS/MS spectrum.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

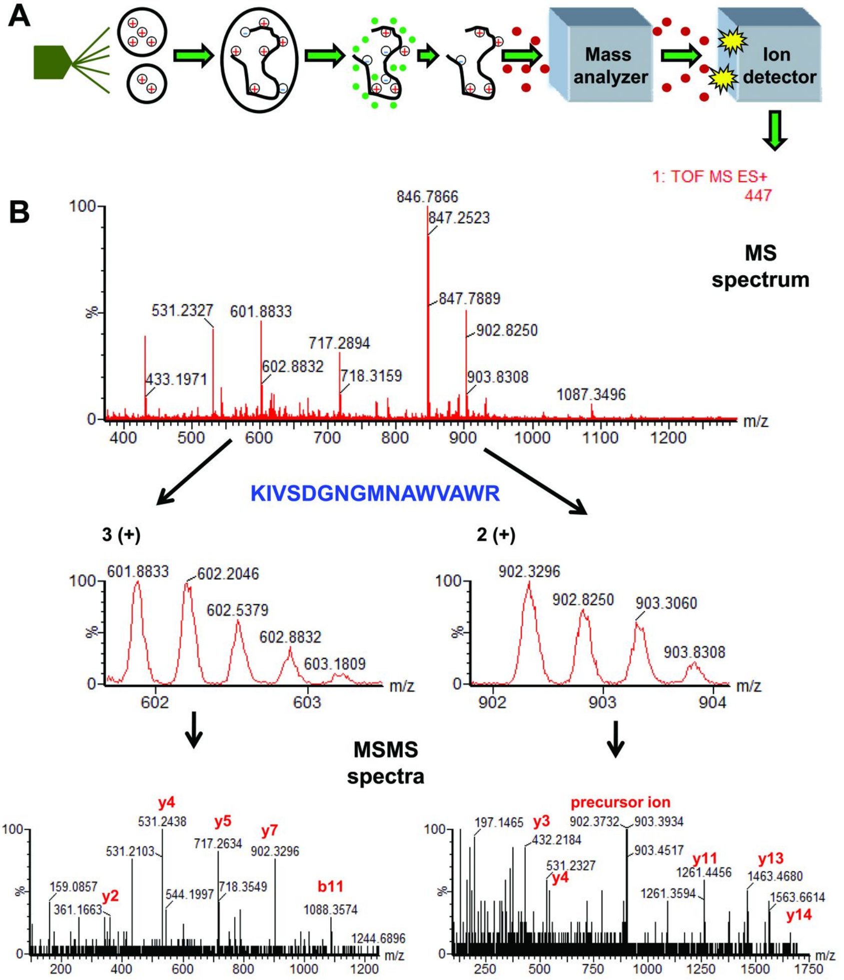


*Figure 3. ESI-MS. **(A)** Schematic of a ESI-MS. The ESI-MS has an ion source, a mass analyzer, and an ion detector, where the mass spectrum is recorded. Unlike in MALDI MS, where the sample is co-crystalized with a matrix, in ESI-MS, the sample is in liquid form that, under high electric current and high temperature (there are also cold spray ESI and nanospray ESI), is dehydrated and becomes protonated. **(B)** Example of a TOF MS spectrum, where two different peaks, one triple charged peak with m/z of 601.88 and one double charged peak with m/z of 902.32 are produced through ionization of the same peptide with the sequence KIVSDGNGMNAWVAWR, which is part of chicken Lysozyme protein (gi:126608). The fragmentation of these MS peaks in MS/MS produced a series of b and y ions that confirmed the identity of the peptide. Both b and y ions result from fragmentation of the precursor ion (that corresponds to the peptide) at the peptidic bond. The ions that correspond to peptidic fragments that contain the N-terminal amino group are named b ions, while the ions that correspond to*

peptidic fragments that contain the C-terminalcarboxyl group are named y ions. Other ions that result from the fragmentation of the precursor ion exist, such as a, c, x, z, w ions, but are not discussed here. Sometimes the MS/MS spectra of two differently charged ions that correspond to the same peptide may either be similar (and the MS/MS spectra confirm each other) or different (and the spectra are complementary with each other). Either way, in both cases, the MS/MS spectra provide sequence information that leads to identification of a protein.

Analysis of the fragment ions (product ions) from the MS/MS spectrum, corroborated with analysis of their parent/precursor ion from MS spectrum (and sometimes from MS/MS spectrum) are then used to identify the sequence of a particular peptide. Fragmentation of the peptides takes place at the peptidic bonds of the peptide, producing mostly fragments of the original peptide; when the fragments contain the N-terminal amino acid, they are named b ions and when the fragments contain the C-terminal amino acid, they are named y ions. Other ions (a, c, x, z, etc.) or internal fragments are also produced in CID. Examples of MS/MS spectra of double and triple charged peaks that correspond to the same peptide are shown in Figure 3B.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Direct Infusion ESI-MS and ESI-MS/MS

Direct infusion is an experiment in which the investigator can quickly check the mass of a compound and/or identify the sequence of that compound (in our case peptide). Because there is no fractionation (by LC), direct infusion experiments are mostly used for analysis of one peptide at a time and is not suitable for automated sequencing. However, this method is very powerful in obtaining sequence information for a particular peptide, especially when the peptide has more than one ion species (double and multiple charged peptides) or when a peptide can be derivatized to obtain additional information about it.

Sometimes, when a peptide has more than one proton accepting amino acid such as Arg or Lys, the peptide may be protonated by more than two or three protons. Therefore, the same peptide may be identified with a higher order of charge state. The advantage for these peptides is that if the precursor peak that corresponds to one charge state (e.g. 2+) does not fragment well in MS/MS, then the peak that corresponds to a different charge state (e.g. 3+ or 4+) may fragment very well. For example, a good fragmentation in CID is when a precursor ion produces a continuous y ion series with additional b ions (with high intensity in the spectrum), and no internal fragments. One drawback for the multiply-charged peptides is that they are usually longer (2500-3000 Da) than the regular peptides analyzed by MS (800-2500 Da) and data analysis for these peptides may be more difficult than for regular peptides. In addition, having a peptide with multiple charges in the MS can also be detrimental due to reduced signal available for MS/MS analysis. However, overall, fragmentation of more than one peak corresponding to the same peptide but with different charge states may help in obtaining additional information about that peptide. Due to the intrinsic property of peptides and proteins to be protonated, researchers can identify the mass and the number of charges in a protein, even if its molecular mass is very high. Very large proteins ionize to yield a series of differently protonated (charged) molecules. This information (delta mass and delta charge) can be used to determine the molecular mass.

LC-MS/MS

When analyzed by ESI-MS, the peptide mixture may also be fractionated before being analyzed by MS. Usually the peptide mixture is fractionated on-line with MS or MS/MS by HPLC on different columns (usually reverse phase HPLC or LC) and different dimensions (1D: reverse phase or 2D in line ion exchange and reverse phase), followed by ESI-MS analysis. Usually the combination of LC and ESI-MS is named LC-ESI-MS or LC-MS. Similarly, LC can be used for MS/MS experiments and the combination is known as LC-MS/MS. LC-MS/MS is also known as tandem MS experiments. Analysis of a peptide mixture of a protein using this approach provides not only the protein identity, but also sequence information for that particular protein. When HPLC is coupled with tandem MS/MS, the LC-MS/MS is more powerful, because the peptide mixture is first loaded on a reverse phase column and then gradually eluted using a mixture of organic eluents. Therefore, the peptides are not analyzed simultaneously, but are analyzed individually (assuming a perfect separation), according to their elution profile from the reverse phase column. The MS and MS/MS analysis then provides masses for proteolytic peptides from a protein digest or from the digest of a complex mixture of proteins and sequence information for the peptides from the MS/MS analyses. A schematic of the LC-MS/MS showing the switch from MS mode to MS/MS mode and back to MS mode is shown in Figure 4A. Overall, a LC-MS/MS experiment will contain a chromatogram (total ion current or TIC) with variable length (10 min-200 minutes). In every specified amount of time (depending on the type of the instrument; e.g. one second) of the experiment, the mass spectrometer will perform a survey scan and record a MS mass spectrum. The most intense peaks in that particular elution time are then selected for MS/MS, fragmented by CID and recorded. The mass spectrometer usually needs a specific amount of time for a MS/MS (e.g. for simplicity, one second per MS/MS). Therefore, if the method is set to have one MS survey scan and then to do MS/MS of the two most intense peaks, then the instrument will work as follows: one second MS survey, one second MS/MS (Peak 1), one second MS/MS (Peak 2) and then again one second MS survey (Figure 4A). However, some mass spectrometers are much faster in analyzing peptide mixtures. For example, some instruments are capable to perform one MS scan and up to 8 MS/MS scans within one second. Assuming the most conservative case (one second per MS, two MS/MS each in one second and then again one second MS scan), we can conclude that in one minute, the mass spectrometer will perform 20 MS surveys and MS/MS of 40 peaks. Data analysis of these MS and MS/MS leads to identification of peptides that are part of proteins.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

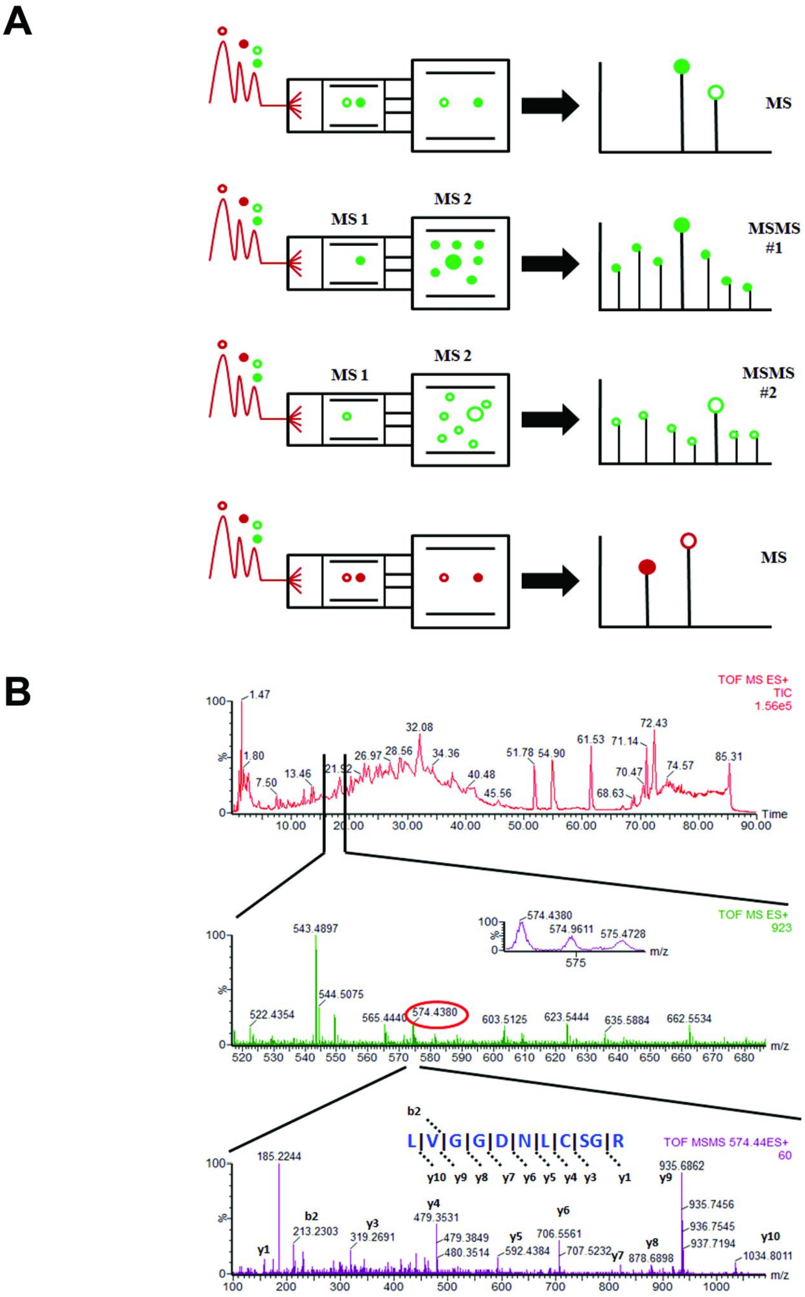


Figure 4. LC-MS/MS experiment. ***(A)*** *In every LC-MS/MS experiment, with the gradual elution of the peptides from the HPLC, the mass spectrometer analyzes the ions that correspond to these peptides by a MS survey (recorded in a MS*

spectrum) and the ions with the highest intensity (usually 1-8 ions; in this example, two ions) are selected for fragmentation by MS/MS, are fragmented and recorded as MS/MS #1 and MS/MS #2. The mass spectrometer then returns to the MS function and records a MS spectrum (MS survey). Again, the ions with the highest intensity are selected for fragmentation, are fragmented and then are recorded as MS/MS spectra. ***(B)*** *Example of a LC-MS/MS experiment where the total ion current is recorded and at a specific time, a MS survey is recorded and one peak that corresponds to a peptide is selected and fragmented in MS/MS. The fragmentation pattern (mostly b and y ions) from MS/MS provides sequence information about the peptide and leads to its identification in a database search. Here, the peptide identified had the sequence LVGGDNLCSGR that was part of human CD5 antigen-like protein (gi:5174411).*

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Assuming that one protein is identified by two peptides and that the MS performs a cycle of one MS and two MS/MS, this means that in one minute, the MS instrument can perform 40- MS/MS that can lead to identification of 20 proteins. Assuming that the length of the chromatographic step is 120 minutes the average number of the proteins that can be identified by data analysis ranges between 200 and 400 proteins. In the other extreme, assuming one MS and 9 MS/MS in one second and identification of a protein by two peptides, then data analysis can lead to identification of four proteins per second and 240 proteins per minute. In a 120 minute gradient, the maximum amount of proteins that can be identified ranges between 14,000-28,000 proteins. However, in these two extreme examples, we assume that every parameter that may interfere or contribute to the experiment (e.g. HPLC time, gradient and flow rate) is constant, but they can dramatically change the outcome of an LC-MS/MS run. For example, when the MS conditions are constant, a 180 minutes linear gradient on the HPLC will lead to analysis of more peptides in the mass spectrometer than a 60 minutes gradient. Assuming constant MS conditions, and constant time of the HPLC method, but with a gradient that is either shallow or sharp, a shallow gradient will lead to analysis of more peptides by MS (and subsequent identification of more proteins). Also, the flow rate may have a high impact in the outcome of the experiment. For example, a flow rate of 100 nanoliters/minute will allow the MS to analyze more peptides per minute, compared with an experiment where the flow rate of the HPLC is 300 nanoliters/minute. The type of the column and optimization of the nanospray also are crucial factors that may heavily influence the outcome of an LC-MS/MS run. In practice, in one good LC-MS/MS run usually leads to identification of 500-1000 proteins. An example of a TIC, MS and MS/MS is shown in Figure 4B.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Data Analysis

The data acquired by a mass spectrometer are usually processed with software (e.g.: Protein Lynx Global Server, PLGS from Waters Corporation) into a peak list or by chromatograph software from other companies into a different output data that is used for database search. There are many database search engines such as Sequest, X!Tandem, Mascot, or Phenyx; here we mostly refer to PLGS for raw data processing and to Mascot search engine (Matrix Science) for database search. The peak list obtained from the MALDI-MS raw data is submitted to the Mascot database for peptide mass fingerprinting, while the peak list obtained from the raw data produced by ESI-MS/MS or LC-MS/MS is submitted to the Mascot search engine for fragment ion search. In both cases, the result is a protein or a list of proteins that are potential hits. In addition to qualitative information provided by MALDI-MS or LC-MS/MS analysis, MS may also provide quantitative information about a particular protein. Methods such as differential gel electrophoresis (DIGE) (*36*), isotope-coded affinity tag (ICAT) (*5*), stable isotope labeling by amino acids in cell culture (SILAC) (*37*), absolute quantitation (AQUA) (*38*), multiple reaction monitoring (MRM) (*39*), or spectral counting (*40*) allow detection, identification and quantification of proteins or peptides. Some of these methods can be specifically applied to the identification and quantitation of tissue and serum biomarkers.

Oxidative Stress

Oxidative stress refers to the generation of reactive oxygen species (ROS), which have the potential to cause cellular and tissue damage. Oxidative stress has been identified as a major pathomechanism possibly involved in a plethora of somatic and neuropsychiatric disorders including cardiopulmonary and metabolic disorders, cancer, alcoholism as well as cerebrovascular and neurodegenerative disorders such as Parkinson and Alzheimer disease (*41–48*). Oxidative stress may be generated by many endogenous sources, including mitochrondia, membrane oxidases, catechols and quinones, products of inflammation and nitric oxide synthase (*49*). Mitochondria function as the “power house” of the cell, generating ATP for energetic processes. In doing so, they may unfortunately generate free radicals (peroxides and hydrogen peroxide are also produced). Mitochondrial dysfunction and free radical generation has been implicated in many disease processes. This includes neurodegenerative disease (*50*, *51*), cardiovascular disease (*52*, *53*) and cancer (*54*) as well as other diseases. Exogenous sources can also generate oxidative stress, including oxidants (such as those found in pesticides or hydrogen peroxide) inducers or xenobiotic quinones (*49*).

Use of Proteomics To Investigate Oxidative Stress

Oxidative stress triggered by reactive oxygen species (ROS) causes various changes in a cell's proteome. Damage can include peptide backbone cleavage by hydroxyl radicals. The side chains of proteins can undergo modifications such as tyrosine hydroxylation, methionine or cysteine oxidation, with methionine oxidation as the most common modification. Another common modification is carbonyl formation on side chain amino acids (*23*). Identification of modified proteins may be used to recognize the levels of damage produced by the oxidative stress. Therefore, those modified proteins can serve as a biomarkers of oxidative stress (*55*). Modified proteins may be identified using so-called redox proteomics approaches (*56*). Because most of changes caused by oxidative stress are post-translational modifications (PTMs), proteomics-based techniques are more suited to the study of these events rather than other global approaches such as genomics or transcriptomics. Cells undergo oxidative stress constituitively, at different levels during their lifetime; therefore most differences in the proteome that are expected are quantitative. Among others, two-dimensional electrophoresis and quantification tools (Western Blotting, DIGE) coupled with mass spectrometry are still common protein characterization tools (*57–59*). Modification of the thiol group of cysteine (which is very sensitive to oxidation) can be monitored using off-diagonal electrophoresis, thiol modifying reagents or more recently *in situ* labeling techniques (*60*, *61*). Knowledge of post-translational modifications induced by oxidative stress is still far from being complete; therefore quantitative changes in subsequently the expression of frequently affected proteins are being addressed (*62–65*). One example of proteins that change during oxidative stress are, among many others, the members of the Heat Shock Protein (HSP) family (*66–68*). Expression levels of the constituents of the Heat Shock Protein (HSP) family are modified by oxidative stress and are therefore of high interest for the proteomic investigation of this process. Expression of HSPs increases when oxidative damage takes place (*66–68*). Pro-oxidative chemicals can induce a protein unfolding response (UPR) which is followed by the induction of HSPs (*69*). Proteomic identification of changes in the expression level of HSPs may be used as a tool to monitor oxidative damage (see below).

Proteomics allows for the examination of proteins that have been altered by oxidative stress (*49*, *70–72*). For example, proteomic profiling was used to analyze the proteins in a triple transgene mouse model of Alzheimer's disease (*73*) and the mitochondrial oxidatively-modified proteins were identified via proteomics in aged mouse brain, specifically in cerebral cortex and hippocampus, areas affected most by aging (*74*). Finally, proteomics have been used to understand how mitochondrial function may alter protein aggregation in neurodegenerative diseases (*75*). Quantitative proteomics is also used to investigate oxidative stress. For example, one proteomic study identified that mitochondrial superoxide dismutase (SOD2; which destroys the normally-produced superoxide anion radicals toxic to cells) and aldehyde dehydrogenase X (ALDH1B; plays an important role in detoxification of alcohol-derived acetaldehyde) were low in patients with ethylmalonic encephalopathy (*76*). In addition, proteomics has the potential to evaluate drug therapies targeted at the prevention of oxidative damage

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

by evaluating protein modifications (*49*). These are just a few examples where proteomics can be applied to investigate oxidative stress, and many are yet to be revealed.

Oxidative Damages of Amino Acids

Proteomic analysis of oxidative stress may be difficult at both the protein identification and protein quantitation levels. Some of the biggest challenges include the large number of amino acids that are potentially modified by simple oxidation and the proteomic consequences of these oxidations. Many amino acids are susceptible to oxidation (*77–86*). Common modifications of amino acids include oxidation of Arginine to Glutamic acid (-27), of Lysine to aminoadipic semialdehyde (-1), of Tryptophan to kynurenine (+4), or of Lysine to aminoadipic acid (+15). Hydroxylation of carbon delta of Lysine, carbon beta of Tryptophan, of carbons C3 or C4 of Proline or of carbon beta of Aspartate (+16) are also common (*77–86*). Other common oxidative modifications include oxidation of Methionine to Sulphoxide (+16), formation of oxohistidine from Histidine (+16) and of sulfenic acid from Cysteine (+16), as well as oxidation of Proline to gamma-glutamyl semialdehyde (+16) and to Glutamic acid (+32), or oxidation of Methionine to Sulphone (+32). Double oxidation of Tryptophan (+32) and oxidation of Cysteine to cysteic acid (+48) are also common (*77–86*). In fact, the double oxidation of Tryptophan to N-formylkynurenine (+32) is almost always accompanied by additional oxidation products of Tryptophan such as kynurenine (+4), an unknown by-product found in all oxidized tryptophan patterns (+13), hydroxytryptophan (+16), 3-hydroxykynurenine (+20) and hydroxy-N-formylkynurenine (+48) (*87*, *88*). Example of MS and MS/MS of a peptide that contains an unmodified Methionine and an oxidized Methionine residue is shown in Figure 5. A summary of the most common oxidative amino acid modifications identified by MS experiments is presented in Table 1.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

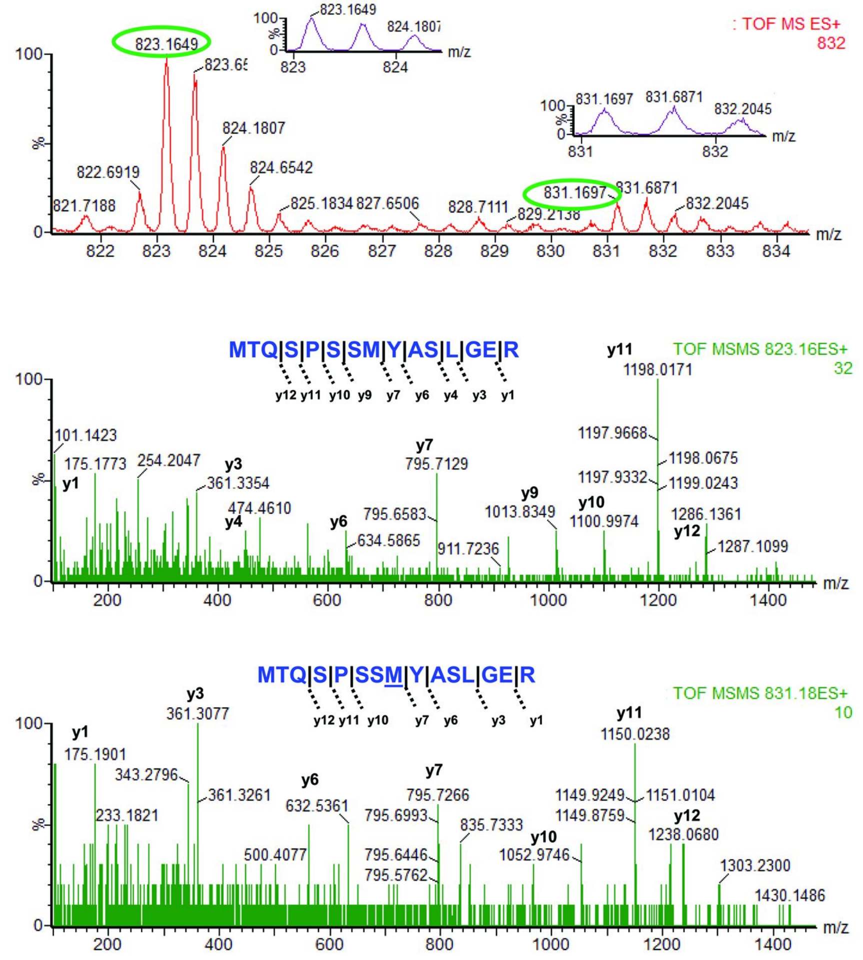


Figure 5. Identification of oxidation of Methionine by LC-MS/MS. A peptide mixture was analyzed by LC-MS/MS. After processing of the raw data and database search, we identified among others, a protein Anti-colorectal carcinoma light chain (from mouse). One of the peptides that is part of this protein, with the sequence MTQSPSSMYASLGER, was identified in the MS spectrum as a double charged peak that corresponds to an ion with m/z of 823.16 ***(A)****. Fragmentation of this ion in MS/MS led to identification of this peptide* ***(B)****. In the MS mode, a second double charged ion with m/z of 831.16 was identified, that corresponded to the same peptide identified previously, with the sequence MTQSPSSMYASLGER, but with one of the underlined Methionines oxidized to Methionine sulphoxide. The difference between the m/z of the two double charged peaks (823.16 versus 831.16), a 16 Da difference, indeed corresponds to the oxidation of one Methionine from the peptide, also identified in the database search. Fragmentation of the double charged peak with m/z of 831.16 by MS/MS confirmed that the MTQSPSSMYASLGER peptide has one Methionine oxidized*

*and also identified the Methionine from the sequence SSMYA (the Methionine from the middle of the peptide) as the modified methionine **(C)**. The difference in the m/z of y10-12 in the two MS/MS spectra (48 Da; **B** and **C**) not only confirms the oxidation of the mentioned Methionine, but also demonstrates that the Methionine underwent oxidation at the Methionine residue (+16 Da) and then suffered a neutral loss of methane-sulfenic acid (loss of 64 Da), which gives us a total difference of 48 Da (observed in **B** and **C**).*

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Table 1. Common Modifications of Amino Acids and Their Products

Amino Acid	*Product of modification*	*Type of modification*	*Change in m/z*
arginine	glutamic acid	oxidation	-27
lysine	aminoadipic semialdehyde	oxidation	-1
lysine	aminoadipic acid	oxidation	+15
methionine	sulphoxide	oxidation	+16
histidine	oxohistidine	oxidation	+16
methionine	sulphone	oxidation	+32
cysteine	sulfenic acid	oxidation	+16
cysteine	cysteic acid	oxidation	+48
proline	gamma-glutyl semialdehyde	oxidation	+16
proline	glutamic acid	oxidation	+32
tryptophan	kynurenine	oxidation	+4
tryptophan	N-formylkynurenine	double oxidation	+32
tryptophan	hydroxytryptophan 3-hydroxykynurenine hydroxyl-N-formylkynurenine unknown by-product	oxidations (accompanying double oxidation)	+16 +20 +48 +13
lysine	5-hydroxylysine	hydroxylation (C5)	+16
tryptophan	3-hydroxytryptophan	hydroxylation (C3)	+16
proline	3-hydroxyproline 4-hydroxyproline	hydroxylation (C3 or C4)	+16
aspartate	3-hydroxyaspartate	hydroxylation (C3)	+16

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Oxidation of Amino Acids Identified in a Proteomics Experiment

As mentioned, the amino acids that are prone to oxidation represent almost half of all amino acids in a protein. Since there are so many amino acids that may be modified, one of the biggest challenges in a proteomics experiment is to identify proteins that contain many modified amino acids. This is not an easy task. If one amino acid is modified in one peptide, then one hopefully can identify an unmodified peptide that will help to identify the protein. However, if more than one amino acid is modified due to oxidative processes, then the chances of identifying that protein become smaller and smaller. In addition, this particular problem increases in small molecular weight proteins, where with decrease of their molecular mass, the chances to be identified becomes even smaller. Furthermore, oxidation of multiple amino acids in small molecular weight proteins makes identification even more difficult. Critical amino acids in proteomics experiments, such as Arginine and Lysine may be changed by oxidative modifications. One of the first problems that one can encounter is that trypsin, the most widely used enzyme in proteomics experiments (it cleaves on the C-terminal side of Arginine and Lysine), will not recognize the cleavage site anymore and will not identify the peptide (and its protein that it belongs to) containing the modified amino acid, but also the peptide downstream of the modified amino acid. Another consequence of amino acid modification is the lack of available bioinformatics tools to identify most of the modified amino acids produced by oxidative stress. Although we mentioned many amino acids that may be modified by partial oxidation, only a few are widely used in database searches and the only true accepted modification is Methionine oxidation. If we compare the oxidation products of Methionine (+16 and +32) with the modification of other amino acids, we run into other problems. For example, hydroxylation of carbon delta of Lysine, carbon beta of Tryptophan, of carbons C3 or C4 of Proline, or of carbon beta of Aspartate mimic Methionine oxidation (+16). Other common oxidative modifications, such as formation of oxohistidine from Histidine (+16) and formation of sulfenic acid from Cysteine (+16), as well as oxidation of Proline to gamma-glutamyl semialdehyde (+16), mimic the oxidation of Methionine to Sulphoxide (+16). In addition, oxidation of Proline to Glutamic acid (+32) and double oxidation of Tryptophan (+32) mimic the oxidation of Methionine to Sulphone (+32). Therefore, if we calculate a (+16) modification and assume that the modification is due to Methionine oxidation (similar for +32 change), we can successfully identify a peptide in MS mode, but encounter problems when one wants to identify the sequence of that peptide, simply because calculating a (+16) or (+32) modification does not guarantee identification of the sequence of that peptide. In fact, it may create more trouble to the researcher through identification of false positive results. Only sequencing the modified peptides by MS/MS can truly identify the correct sequence of that particular peptide and sometimes even identify the amino acid that was oxidized and modified. Therefore, the challenge of identifying a protein or a peptide shifts from the experimental part of the proteomics experiment to the computer-based, bioinformatics portion of the proteomics experiment.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Oxidative Stress and the Analysis of Redox Systems

Perhaps one of the greatest indicators of oxidative stress in a proteomics experiment can be observed via analysis of the reduced and oxidized states of proteins. There are two main approaches: to analyze the redox state of the proteins such as analysis of cytochromes (e.g. cytochrome c or cytochrome b6f), which is usually a proteomics-independent experiment or to analyze the redox state of Cysteine and/or proteins that contain Cysteines in either reduced or oxidized form. The significance of the redox state of Cysteine is crucial at many levels of the protein structure. For example, secreted proteins contain disulfide bridges and if more than two Cysteines from the same protein are involved in disulfide bridges, the correct disulfide linkage may be critical for the physiological function of that protein. As a consequence, if the protein with the correct disulfides can perform its physiological function, the protein that does not have the correct disulfides may not be able to perform its normal function, or even worse: it may be detrimental to its surroundings. Similar rules also apply for disulfide-linked proteins that are connected either as homo- or hetero-dimers or multimers. Measuring the redox state in these proteins means making sure these proteins are in the correct disulfide linkage (which must be known ahead of time) and/or in the correct ratio between the reduced and oxidized protein(s). One way of analyzing disulfide linkages in these proteins is by using mass spectrometry (both MALDI MS and LC-MS/MS). An example of a disulfide assignment is shown in Figure 6.

Heat Shock Proteins

Environmental stress greatly affects all living cells through changes in the synthesis of particular proteins. Different sets of proteins will be preferentially over-expressed or under-expressed under various stress conditions. One widely-studied group of proteins are the HSP family of proteins that respond to stress induced by temperature elevation (*89*, *90*). HSPs are ubiquitous and highly-conserved proteins found in almost all organisms and play an essential role as molecular chaperones by assisting in the correct folding of nascent proteins or proteins that accumulate following a stress response (*91*). Under stress conditions such as heat shock, metals, viral infections, inflammation, ischemia, oxidant injury, malignancy, a higher level of HSP expression is observed that stabilizes misfolded or unfolded proteins, giving a cell more time to re-synthesize damaged proteins (*92*).

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

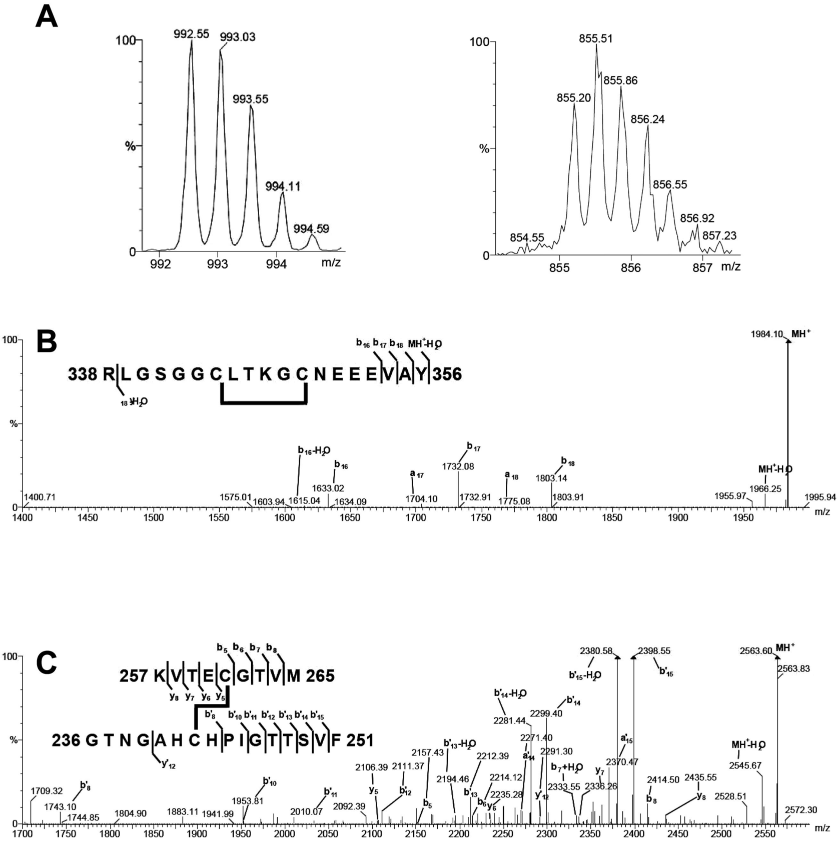


Figure 6. Assignment of disulfide bridges within Vitelline envelope protein beta by LC-MS/MS. The peptide mixture was digested by chymotrypsin and analyzed by LC-MS/MS. The sequences of the identified disulfide-linked peptides are shown. ***(A)*** *A double charged ion with m/z of 992.55 was selected for fragmentation and its MSMS is shown in* ***(B)****. Data analysis led to identification of the peptide shown in* ***(B)****, with two Cysteine residues oxidized in an intramolecular disulfide bridge. A triple charged ion shown in* ***(A)*** *with m/z of 855.20 was selected for fragmentation and its MSMS is shown in* ***(C)****. Data analysis led to identification of two peptides shown in* ***(C)****, with their Cysteine residues oxidized in an intermolecular disulfide bridge. In the intermolecular disulfide bridge, two different peptides are connected with each other through their Cysteine residues. In this experiment, because we used chymotrypsin digestion, the peptides do not end with the regular Arginine or Lysine residues. In addition, while the regular peptides fragment well in the MS/MS and contain only one b and one y series of ions, in this experiment we simultaneously analyzed two different peptides that were disulfide-linked and that contained two different series of b and y ions.*

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Function of HSPs

HSPs are involved in the folding of nascent proteins, protein assembly and transport across membranes, protein turnover/degradation, cell signaling and regulation of transcription factors. Synthesis of HSP under normal physiological conditions depends on the levels of Heat Shock Factor (HSF). Under physiological conditions HSF is an inactive monomer in complex with HSP90 and HSP70 chaperones. Cell stress induces an increase in protein denaturations which triggers release of HSF from the complex and its translocation into the nucleus, where it acts as a transcription factor for HSP genes (*93*, *94*). Members within HSP families include isoforms which are constitutively expressed, such as HSC70 and the previously mentioned HSP90β and inducible isoforms that are up-regulated during stress. Levels of inducible HSP, such as most HSP70 or HSP27, elevates after exposure to stress (*95*). Constitutive expression of HSPs is regulated by affinity to substrates and concentration of denatured proteins. Aberrant expression is linked with various physiological conditions including cancer, cardiovascular diseases, neurological disorders, inflammatory conditions and infectious diseases. Therefore increased expression of many HSPs is often associated with tumorigenesis. For example, HSP70 and HSP27 are involved in tumor resistance due to their anti-apoptotic properties (*96–99*). HSP27 protects against apoptotic death by interactions with various components of apoptotic pathways. It inhibits release of cytochrome *c* from mitochondria which prevents activation of caspases, (main apoptosis executors) (*100*, *101*) and acts as a negative regulator of pro-apoptotic Smac release (*102*).

Characterization of HSPs

HSPs account for approximately 5-10% of all cellular proteins and are classified by their molecular weight into several families: HSP100, HSP90, HSP70/40, HSP60 (chaperonins) and sHSP (small HSP) with molecular weight 8.5-40kDa (*95*, *103*).

HSP90 Proteins

Proteins from the HSP90 family are the most abundant of the all HSPs and are localized localized in the cytosol and nucleus. In proteins from the HSP90 family structure, conserved domains can be distinguished in the C and N-terminus regions, with the ATP-binding and nucleotide-binding domain localized near the N-terminus and the dimerization domain in the C-terminus (*104*, *105*). Monomers of HSP90 proteins bind to form functional dimers through conformational changes induced by binding of ATP and accessory proteins (*106*, *107*). Active HSP90 proteins are involved in cell survival and apoptosis through interaction with a variety of proteins responsible for growth and development, allowing for adaptation to stress conditions and the selection of the strongest cell lineage (*108*). The most-studied members of this family are HSP90α, HSP90β and 94 kDa glucose-regulated protein (GRP94). Expression of HSP90β is believed to be constituitive through a cell's lifetime. This protein is involved in early development, signal transduction and stabilization of the cytoskeleton through interaction with tubulin and actin (*109*). HSP90α expression is inducible by malignant tumor progression and growth factors via tyrosine kinase or nicotine receptors (*104*). Commonly, HSP90β is believed to be responsible for long-term cellular adaptation, whereas HSP90α acts as a fast-responding cellular protector. GRP94 is a major regulator in the endoplasmatic reticulum. It targets proteins for degradation associated with the ER, assists in protein folding in the ER and binds calcium for storage buffering in the ER (*110*).

HSP70 Proteins

HSP70 family members are the best known among HSPs with 8 members already widely characterized. The family of HSP70 is by far the most conserved family of proteins with a domain structure consisting of a C-terminal region binding co-chaperones, a peptide binding domain, a middle region with a protease sensitive site and an N-terminal ATPase domain (*95*, *111*). They are mostly found in the cytosol and nucleus with the exception of HSP70-5/GRP78 (glucose regulated protein 78 kDa), which is responsible for the transport of synthesized proteins into the lumen of the ER and their subsequent folding (*112*) and of HSP70-9/GRP75/mortalin that is mainly found in mitochondria. Proteins not localized in the cytosol have additional specific signal sequences that target them into the ER or mitochondria. The main function of HSP70 proteins is *de novo* folding of nascent polypeptides, but they play important role in many other processes. HSP70-1 (a and b) proteins are expressed in a cell cycle-dependent manner with an increased level between the G1 and S phases of the cell cycle (*113*). Stress-induced members of HSP70-1 proteins play a cytoprotective role, helping degrade the accumulated aggregation of denatured proteins. HSP70-2 is expressed in all tissues but especially high levels are observed in brain tissues, where it prevents accumulation of incorrectly folded proteins responsible for development of neurodegenerative disorders such as Alzheimer's or Parkinson's (*114*). HSP70-6 is an exclusively stress-induced protein that plays a cytoprotective role. HSP70-8 (HSC70) is responsible for protein transport across membranes and chaperone-mediated autophagy. During stress conditions they prevent accumulation of denatured proteins together with HSP70-1 (*115*). HSP70 proteins are aided by HSP40 which increase the ATP rate consumption and activity of HSP70. HSP40 proteins stabilize interactions of HSP70 with their substrates (*116*).

HSP60 Proteins

Chaperone HSP60/10 family members (also called Chaperonins) are localized mainly in the mitochondria but also in the cytosol and cell membrane. HSP60, in cooperation with HSP10, can assist nascent proteins to achieve their native conformation. HSP10 increases the ATPase activity of HSP60. The expression of HSP60 proteins is high under normal conditions and levels do not increase

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

significantly after heat shock (*117*). It was also found that HSP60 proteins play a role in the immune response (*118*, *119*)

Small HSP Proteins

The small HSP protein family has 10 known members which are distinguished from other HSP proteins due to the lack of ATPase domain in their structure. Under normal conditions they occur as large inactive oligomers . Upon heat shock activation they disintegrate into smaller oligomers and become activated to bind substrates and create complexes. They work mainly in the cytosol and nuclear membrane where they maintain proteins homeostasis by transmission of damaged (non-native) proteins to reconstructive HSP70/40 chaperones (*121*, *122*).

Function of HSPs

HSPs are involved in the folding of nascent proteins, protein assembly and transport across membranes, protein turnover/degradation, cell signaling and regulation of transcription factors. Synthesis of HSP under normal physiological conditions depends on the levels of Heat Shock Factor (HSF). Under physiological conditions HSF is an inactive monomer in complex with HSP90 and HSP70 chaperones. Cell stress induces an increase in protein denaturations which triggers release of HSF from the complex and its translocations into nucleus where it acts as a transcription factor for HSP genes (*93*, *94*). Members within HSP families include isoforms which are constitutively expressed, such as HSC70 and previously mentioned HSP90β and inducible isoforms that are up-regulated during stress conditions, whereas level of inducible HSP like most of HSP70 or HSP27 elevates after exposure to stress (*95*). Constitutive expression of HSPs is regulated by affinity to substrates and concentration of denatured proteins. Aberrant expression is linked with various physiological conditions including cancer, cardiovascular diseases, neurological disorders, inflammatory conditions and infectious diseases. Therefore increased expression of many HSPs is often associated with tumorigenesis. For example, HSP70 and HSP27 are involved in tumor resistance due to their anti-apoptotic properties (*96–99*). HSP27 protects against apoptotic death by interactions with various components of apoptotic pathways. It inhibits release of cytochrome *c* from mitochondria which prevents activation of caspases, (main apoptosis executors) (*100*, *101*) and acts as a negative regulator of pro-apoptotic Smac release (*102*).

Roles of HSP70 Proteins

HSP70 family members play an important role in tumor resistance to apoptotic processes. For example, induced HSP70-1 blocks upstream caspase activities, inhibits downstream release of cytochrome c and decreases levels of

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

mitochondrial damage and binding to AIF (apoptosis-inducing factor). It can also block apoptosis at the death receptor level, by binding to them and inhibiting death-inducing signals (*123–125*). Regulation of apoptosis by HSP70 involves many levels of this complicated process, indicating that it is one of the most decisive negative regulators of cell death. A variety of roles for HSP70 proteins in general and of GRP78/BiP in particular is also described elsewhere (*126–137*).

A novel role for members of HSP70 family is as receptors for a recently discovered pituitary factor. Tumor Differentiation Factor (TDF) is a pituitary factor (*138*, *139*), yet to be recognized as hormone, with no known function, mechanism of action, or organ target. Although it has been a while since its discovery, no advancement in deciphering its function has been made since. It is also not known where this protein goes and to what receptor it binds. Current work in our laboratory identified, using affinity chromatography and mass spectrometry, the members of HSP family as potential TDF receptors (*140*). Specifically, we identified GRP78, HSP70-Protein 1 and HSP70-Protein 8, all members of HSP70 family. Therefore, in addition to the variety of functions of HSPs, we added a new one: as potential hormone receptors, specifically for TDF.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Roles of HSP90 Proteins

HSP90 can be up-regulated 10 fold in tumor cells suggesting its role in tumor growth and survival by inhibition of apoptosis and stabilization of various growth factors (*141*, *142*). In tumor cells HSP90 exists entirely in their active multi-complex (*143*). Up-regulation of its levels was detected in hepatocellular carcinoma, leukemia, neuroblastoma and many other carcinomas (*144–147*). In addition to a HSP90 role in tumorigenesis, it was also found that during fungal infections, this chaperone helps in the development of resistance to antifungal agents (*148*, *149*).

Roles of HSP60 Proteins

HSP60 has the potential to be used in the diagnosis of cancer. HSP 60 presence in blood is associated with inflammatory conditions (*150*). A dual function of HSP60 as a biomarker and immune modulator has been proposed (*151*).

Expression of HSPs

Under normal conditions HSPs accumulate in sites of proteins synthesis (endoplasmatic reticulum - ER), mitochondria and in the cytosol. It has been observed that levels of ROS leads to activation of HSPs (*152*). Under pathological conditions (such as tumorigenesis) they can exist on the surface of the cell or in the extracellular space where they have an immunogenic function and play a role in oncogenic cell signaling (*111*, *153–157*). One of the main functions that

HSP proteins play when localized outside the cell is co-presentation of antigenic peptides derived to MHC class I molecules, providing a signal of existing danger to the host immune system (*158*). Oxidative stress is one of the important stress factors that increase the expression of HSPs. An increase in the expression of HSPs can protect against oxidative damage (*66*, *67*). With cellular aging, levels of oxidatively-damaged proteins increase, which competitively bind to HSP instead of HSF. Chaperones are also damaged during the process of aging and are not able to effectively fulfill their functions of preventing cellular aging (*93*, *159*). Members of the HSP family are known to be involved in antioxidant protection of the cell. They induce decreases in ROS through increases in cell glutathione levels. HSP27 is often referred to as a marker of oxidative stress (*153*, *160*, *161*). HSP27 also works as a neutralizer of the toxic effect caused by oxidized proteins (*162*, *163*). Proteins from the HSP60 family have also been observed to act as protectors after exposure to oxidative stress through an anti-apoptotic function (*164*). Plants also respond to oxidative stress by regulation of HSP expression (*165*, *166*).

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Expression of GRP78 and HSP70s

Expression of HSPs might be identified using a proteomics approach involving two dimensional electrophoresis followed by tandem mass spectrometry, MALDI or Western Blotting analysis (*167–169*). An example of an MS and MS/MS spectrum of peptides leading to the identification of HSP70 family member (GRP78) is presented in Figure 7. Once a protein is identified and characterized using mass spectrometry, validation of the results obtained by these methods is imperious. For example, when a protein or a peptide from this protein is determined to be phosphorylated, validation can be easily performed by Western blotting or other similar immunoblotting techniques (e.g. ELISA). Similarly, expression of HSPs can be monitored using immunoblotting techniques (*170*, *171*), differential gel electrophoresis (2D-DIGE) (*25*, *172*, *173*) or immunofluorescence (IF; staining of cells employs antibodies against proteins of interest). Of all these methods, IF is particularly important, since it not only confirms the existence of a protein in a sample and validates an MS experiment, but also immunolocalizes the proteins at the cellular level, allowing the researcher to expand the interpretation of MS experiments beyond a simple validation. An example of an IF experiment using antibodies against HSP70 and GRP78 (both members of the HSP70 family) is presented in Figure 8. Cancerous HeLa cells demonstrate much stronger expression of both HSP70 proteins than normal human dermal fibroblasts (HDF-a). Membrane localization of HSP in HeLa cells can also be observed. Standard proteomics approaches combined with immunoblotting and IF experiments are also great tools for investigating changes of HSP expression profiles following oxidative stress.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

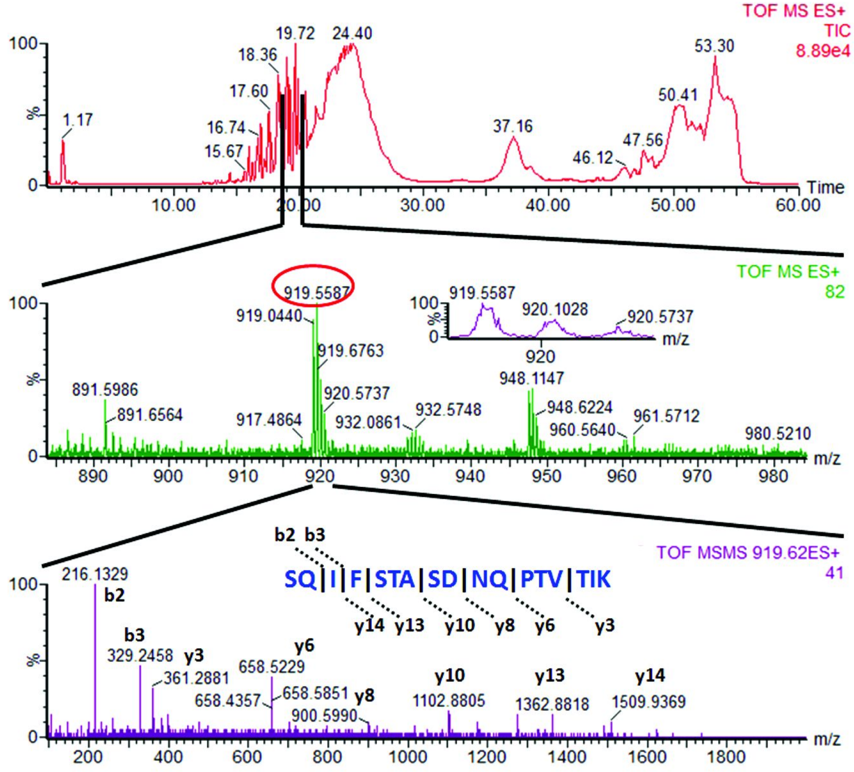


Figure 7. LC-MS/MS analysis of a peptide mixture that lead to identification of HSP70 family member (GRP78). ***(A)*** *TIC of the chromatogram* ***(B)*** *MS mass spectrum with one double charged peak at m/z of 919.55 (expanded in the inbox) that corresponds to peptide with sequence SQIFSTASDNQPTVTIK wich is part of glucose regulated protein GRP78/BiP protein. MSMS fragmentation spectrum produced a series of y and b ions (****C****, marked) that led confirmed the identity and the sequence of the SQIFSTASDNQPTVTIK peptide.*

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

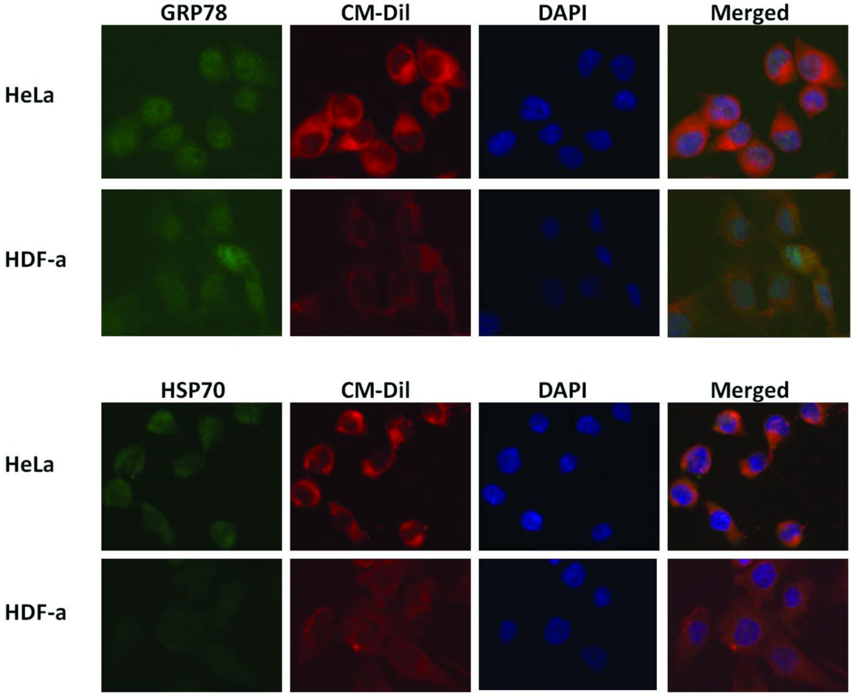


Figure 8. IF detection of HSP70 and GRP78 proteins in cancerous HeLa cells and normal human dermal fibroblasts (HDF-a). HeLa and HDF-a cells were grown on coverslides and incubated with GRP78 or HSP70 primary antibodies, followed by incubation with Alexa Fluor 488-conjugated secondary antibody. Expression of HSP70 and GRP78 proteins is shown in green. Cells were also stained for plasma membrane with CM-Dil (red), and the nuclei were stained with DAPI (blue).

Concluding Remarks

There is much that remains to be investigated about oxidative stress at the protein level. A simple PubMed search using “oxidative stress” as a key phrase revealed 89,532 publications, 76,000 of which were published since 2000 and 17,000 since 2010, suggesting that oxidative stress is a research area of increasing importance (Figure 9). An interesting trend can also be observed regarding mass spectrometry. A search using “mass spectrometry” as a search parameter revealed 172,270 publications, 120,000 of which were published since 2000 and 28,000 since 2010, suggesting that mass spectrometry use is also on the rise. Furthermore, a search using “proteomics” as a term revealed only 30,312 publications, 30,221 of which were published since 2000 and 10,000 since 2010, suggesting that proteomics is perhaps one of the newer disciplines. A search using “oxidative stress and mass spectrometry” as parameters revealed only 2495 publications, of which one fourth (581) were published since 2010. A final search of "oxidative stress and proteomics" revealed only 1145 publications, of which one third (321) were published since 2010.

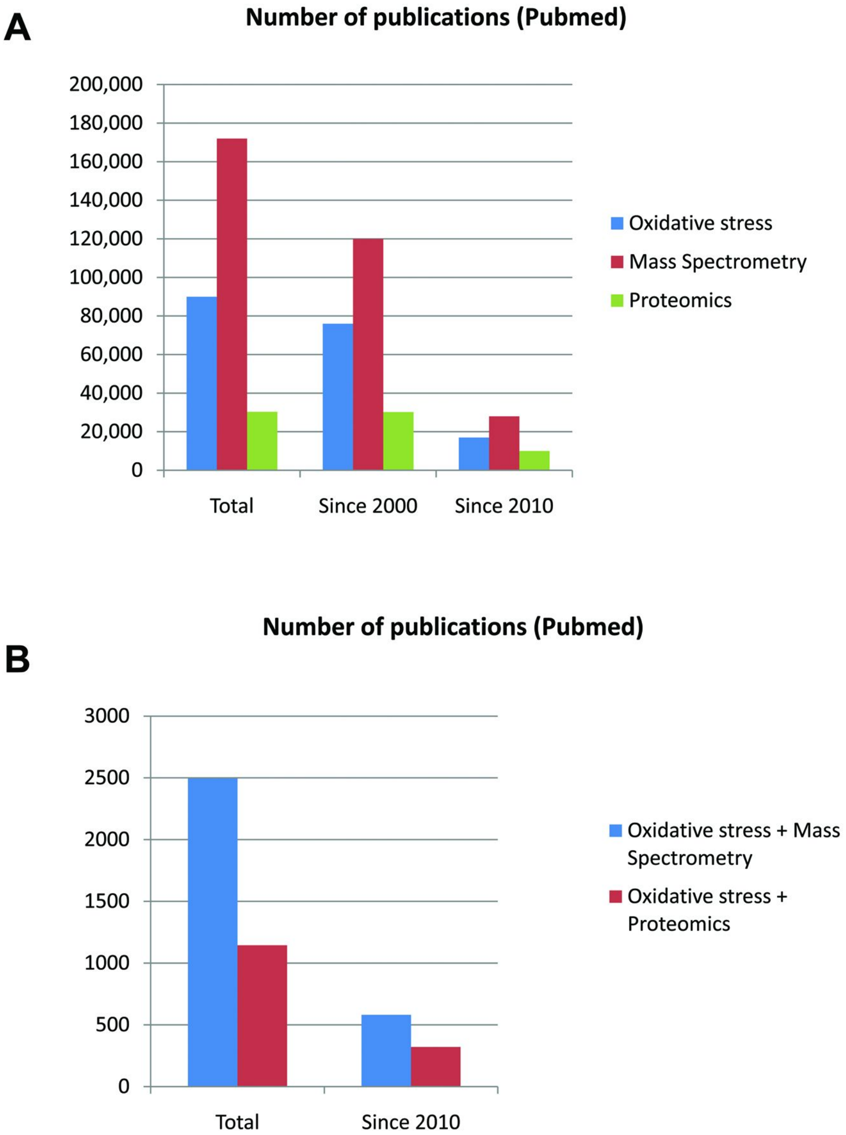


*Figure 9. Pubmed database search using as search keyword oxidative stress, mass spectrometry or proteomics **(A)**. Pubmed database search using as search keywords a combination of oxidative stress and mass spectrometry or oxidative stress and proteomics **(B)**. The number of publications are either from all times, since 2000 or since 2010. Database search is as of 07/27/2011.*

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Overall these searches indicate that mass spectrometry and proteomics have become very useful tools for investigating oxidative stress. There was a relatively high number of publications within the past year about oxidative stress (17,000 publications since 2010), mass spectrometry (28,000 publications since 2010) and proteomics (10,000 publications since 2010), but a relatively small number of papers that resulted when the search parameters used were "oxidative stress and mass spectrometry" (581 published papers since 2010) or "oxidative stress and proteomics" (321 published papers since 2010). This suggests that mass spectrometry in general and proteomics specifically are being increasingly used to investigate oxidative stress.

Further to this PubMed search indicating that oxidative stress is an intriguing topic of research, it is becoming increasingly clear that oxidative stress is an important consideration to applied clinical work. As mentioned previously, oxidative stress is implicated in many medical conditions (*41–48*). Applied proteomic investigation of oxidative stress in disease has the potential to reveal the pathophysiologies of various disorders, and may allow for the identification of clinically relevant biomarkers (*174*). This in turn could aid in disease prevention via identifying oxidative stress targets and may also enable the development of new medications for disease treatment. Applied proteomics—particularly for the investigation of oxidative stress—is therefore set to take a pivotal role in both basic and clinical research, enabling the advancement of medicine.

Abbreviations

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mw, molecular weight; DIGE, differential gel electrophoresis; MS, mass spectrometry; FAB, fast atom bombardment; PD, plasma desorption; EI, electric ionization; CI, chemical ionization; ESI, electrospray ionization; MALDI, Matrix Assisted Laser Desorption Ionization; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, MALDI mass spectrometry; LC-MS/MS, liquid chromatography mass spectrometry; m/z, mass/charge; CID, collision-induced dissociation; TIC, total ion current/chromatogram; TOF, time of flight; IT, ion trap, FT, Fourier transform; Q, quadrupole; BN-PAGE, Blue Native PAGE; CN-PAGE, Colorless Native PAGE; HSP, Heat shock protein; HSP, Heat Shock Factor; GRP78, Glucose Receptor Protein 78; TDF, Tumor Differentiation Factor, HDF-a, normal human dermal fibroblasts; IF, Immunofluorescence microscopy.

Acknowledgments

The authors would like to thank Laura Mulderig, Todd Boyer, Scott Nichols, Nathan Vanderlan, Tim Mascal, Keith Compson, Michael Balloch, Catalin Doneanu, David Chamberlin, Cathy Bolland, Joanne Boland, Janet Gallager, Sean McCarthy, Cristian Cojocariu and Charles Pragnell from Waters Corporation for the collaborative discount that allowed us to purchase a Micromass Quadrupole Time of Flight (QTOF) Micro mass spectrometer. The authors would also like to thank Thomas A. Neubert from the Skirball Institute, New York University for donation of the TofSpec2E MALDI-TOF mass spectrometer and Steven Blais (Skirball Institute, New York University) and Jason Watson, Mark Welsh and Tom Adams (Waters Corporation) for technical support and advice in setting up the MALDI MS. The authors also thank Drs. Thomas A. Neubert, Paul M. Wassarman, Eveline Litscher, Wolfgang Haehnel, Vlad Artenie and Kenneth R. Woods for advice and support. This work was partially supported by Clarkson University (start-up to C.C.D.) and U.S. Army Research Office (DURIP program) through grant #W911NF-11-1-0304.

References

1. Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422*, 198–207.
2. Aivaliotis, M.; Karas, M.; Tsiotis, G. High throughput two-dimensional blue-native electrophoresis: A tool for functional proteomics of cytoplasmatic protein complexes from *Chlorobium tepidum*. *Photosynth. Res.* **2006**, *88*, 143–157.
3. Blagoev, B.; Kratchmarova, I.; Ong, S. E.; Nielsen, M.; Foster, L. J.; Mann, M. A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat. Biotechnol.* **2003**, *21*, 315–318.
4. Camacho-Carvajal, M. M.; Wollscheid, B.; Aebersold, R.; Steimle, V.; Schamel, W. W. Two-dimensional Blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: A proteomics approach. *Mol. Cell. Proteomics* **2004**, *3*, 176–182.
5. Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17*, 994–999.
6. Ong, S. E.; Foster, L. J.; Mann, M. Mass spectrometric-based approaches in quantitative proteomics. *Methods* **2003**, *29*, 124–130.
7. Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850–858.
8. Zhang, G.; Spellman, D. S.; Skolnik, E. Y.; Neubert, T. A. Quantitative phosphotyrosine proteomics of EphB2 signaling by stable isotope labeling with amino acids in cell culture (SILAC). *J. Proteome Res.* **2006**, *5*, 581–588.
9. Darie, C. C.; Biniossek, M. L.; Winter, V.; Mutschler, B.; Haehnel, W. Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*. *FEBS J.* **2005**, *272*, 2705–2716.
10. Darie, C. C.; Janssen, W. G.; Litscher, E. S.; Wassarman, P. M. Purified trout egg vitelline envelope proteins VEbeta and VEgamma polymerize into homomeric fibrils from dimers in vitro. *Biochim. Biophys. Acta* **2008**, *1784*, 385–392.
11. Schagger, H.; Cramer, W. A.; von Jagow, G. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **1994**, *217*, 220–230.

12. Schagger, H.; von Jagow, G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **1991**, *199*, 223–231.
13. Spellman, D. S.; Deinhardt, K.; Darie, C. C.; Chao, M. V.; Neubert, T. A. Stable isotopic labeling by amino acids in cultured primary neurons: application to brain-derived neurotrophic factor-dependent phosphotyrosine-associated signaling. *Mol. Cell. Proteomics* **2008**, *7*, 1067–1076.
14. Darie, C. C.; Shetty ,V.; Spellman D. S.; Zhang G.; Xu C.; Cardasis H. L.; Blais S.; Fenyo D.; Neubert, T. A. *Blue Native PAGE and Mass Spectrometry Analysis of the Ephrin Stimulation- Dependent Protein-Protein Interactions in NG108-EphB2 Cells*; Springer-Verlag: Düsseldorf, Germany, 2008.
15. Darie, C. C.; Litscher E. S.; Wassarman P. M. *Structure, Processing, And Polymerization of Rainbow Trout Egg Vitelline Envelope Proteins*; Springer-Verlag: Düsseldorf, Germany, 2008.
16. Darie, C. C.; Biniossek, M. L.; Gawinowicz, M. A.; Milgrom, Y.; Thumfart, J. O.; Jovine, L.; Litscher, E. S.; Wassarman, P. M. Mass spectrometric evidence that proteolytic processing of rainbow trout egg vitelline envelope proteins takes place on the egg. *J. Biol. Chem.* **2005**, *280*, 37585–37598.
17. Darie, C. C.; Biniossek, M. L.; Jovine, L.; Litscher, E. S.; Wassarman, P. M. Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. *Biochemistry* **2004**, *43*, 7459–7478.
18. Schagger, H. Tricine-SDS-PAGE. *Nat. Protoc.* **2006**, *1*, 16–22.
19. Schagger, H.; von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368–379.
20. Jovine, L.; Darie, C. C.; Litscher, E. S.; Wassarman, P. M. Zona pellucida domain proteins. *Annu. Rev. Biochem.* **2005**, *74*, 83–114.
21. Litscher, E. S.; Janssen, W. G.; Darie, C. C.; Wassarman, P. M. Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions. *J. Cell. Physiol.* **2008**, *214*, 153–157.
22. Wassarman, P. M.; Jovine, L.; Qi, H.; Williams, Z.; Darie, C.; Litscher, E. S. Recent aspects of mammalian fertilization research. *Mol. Cell. Endocrinol.* **2005**, *234*, 95–103.
23. Ghezzi, P.; Bonetto, V. Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* **2003**, *3*, 1145–1153.
24. Li, X.; Pan, W.; Yang, G. Z.; Di, Y. N.; Zhao, F.; Zhu, L. Y.; Jiang, Z. H. Proteome analysis of differential protein expression in brain of rats with type 1 diabetes mellitus. *Exp. Clin. Endocrinol. Diabetes* **2011**, *119*, 265–270.
25. Muroi, M.; Kazami, S.; Noda, K.; Kondo, H.; Takayama, H.; Kawatani, M.; Usui, T.; Osada, H. Application of proteomic profiling based on 2D-DIGE for classification of compounds according to the mechanism of action. *Chem. Biol.* **2010**, *17*, 460–470.
26. Polden, J.; McManus, C. A.; Remedios, C. D.; Dunn, M. J. A 2-D gel reference map of the basic human heart proteome. *Proteomics* **2011**.

27. Stefanescu, R.; Iacob, R. E.; Damoc, E. N.; Marquardt, A.; Amstalden, E.; Manea, M.; Perdivara, I.; Maftei, M.; Paraschiv, G.; Przybylski, M. Mass spectrometric approaches for elucidation of antigenantibody recognition structures in molecular immunology. *Eur. J. Mass Spectrom.* **2007**, *13*, 69–75.
28. Sun, X.; Jia, H. L.; Xiao, C. L.; Yin, X. F.; Yang, X. Y.; Lu, J.; He, X.; Li, N.; Li, H.; He, Q. Y. Bacterial proteome of *Streptococcus pneumoniae* through multidimensional separations coupled with LC-MS/MS. *OMICS* **2011**.
29. Wang, Y.; Li, R.; Du, D.; Zhang, C.; Yuan, H.; Zeng, R.; Chen, Z. Proteomic analysis reveals novel molecules involved in insulin signaling pathway. *J. Proteome Res.* **2006**, *5*, 846–855.
30. Bauw, G.; Rasmussen, H. H.; van den Bulcke, M.; van Damme, J.; Puype, M.; Gesser, B.; Celis, J. E.; Vandekerckhove, J. Two-dimensional gel electrophoresis, protein electroblotting and microsequencing: a direct link between proteins and genes. *Electrophoresis* **1990**, *11*, 528–536.
31. Celis, J. E.; Gromov, P. 2D protein electrophoresis: Can it be perfected? *Curr. Opin. Biotechnol.* **1999**, *10*, 16–21.
32. Celis, J. E.; Gromov, P.; Ostergaard, M.; Madsen, P.; Honore, B.; Dejgaard, K.; Olsen, E.; Vorum, H.; Kristensen, D. B.; Gromova, I.; Haunso, A.; Van Damme, J.; Puype, M.; Vandekerckhove, J.; Rasmussen, H. H. Human 2-D PAGE databases for proteome analysis in health and disease: http://biobase.dk/cgi-bin/celis. *FEBS Lett.* **1996**, *398*, 129–134.
33. Celis, J. E.; Gromova, I.; Moreira, J. M.; Cabezon, T.; Gromov, P. Impact of proteomics on bladder cancer research. *Pharmacogenomics* **2004**, *5*, 381–394.
34. Taurines, R.; Dudley, E.; Conner, A. C.; Grassl, J.; Jans, T.; Guderian, F.; Mehler-Wex, C.; Warnke, A.; Gerlach, M.; Thome, J. Serum protein profiling and proteomics in autistic spectrum disorder using magnetic bead-assisted mass spectrometry. *Eur. Arch. Psychiatry Clin. Neurosci.* **2010**, *260*, 249–255.
35. Taurines, R.; Dudley, E.; Grassl, J.; Warnke, A.; Gerlach, M.; Coogan, A. N.; Thome, J. Proteomic research in psychiatry. *J. Psychopharmacol.* **2011**, *25*, 151–196.
36. Viswanathan, S.; Unlu, M.; Minden, J. S. Two-dimensional difference gel electrophoresis. *Nat. Protoc.* **2006**, *1*, 1351–1358.
37. Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **2002**, *1*, 376–386.
38. Stemmann, O.; Zou, H.; Gerber, S. A.; Gygi, S. P.; Kirschner, M. W. Dual inhibition of sister chromatid separation at metaphase. *Cell* **2001**, *107*, 715–726.
39. Anderson, L.; Hunter, C. L. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **2006**, *5*, 573–588.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

40. Liu, H.; Sadygov, R. G.; Yates, J. R., 3rd A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76*, 4193–4201.
41. Essick, E. E.; Sam, F. Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer. *Oxid. Med. Cell. Longevity* **2010**, *3*, 168–177.
42. Hayashi, M. Oxidative stress in developmental brain disorders. *Neuropathology* **2009**, *29*, 1–8.
43. Jomova, K.; Vondrakova, D.; Lawson, M.; Valko, M. Metals, oxidative stress and neurodegenerative disorders. *Mol. Cell. Biochem.* **2010**, *345*, 91–104.
44. Kahle, P. J.; Waak, J.; Gasser, T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. *Free Radical Biol. Med.* **2009**, *47*, 1354–1361.
45. Migliore, L.; Coppede, F. Environmental-induced oxidative stress in neurodegenerative disorders and aging. *Mutat. Res.* **2009**, *674*, 73–84.
46. Thome, J.; Foley, P.; Gsell, W.; Davids, E.; Wodarz, N.; Wiesbeck, G. A.; Boning, J.; Riederer, P. Increased concentrations of manganese superoxide dismutase in serum of alcohol-dependent patients. *Alcohol Alcohol* **1997**, *32*, 65–69.
47. Thome, J.; Gsell, W.; Rosler, M.; Kornhuber, J.; Frolich, L.; Hashimoto, E.; Zielke, B.; Wiesbeck, G. A.; Riederer, P. Oxidative-stress associated parameters (lactoferrin, superoxide dismutases) in serum of patients with Alzheimer's disease. *Life Sci.* **1997**, *60*, 13–19.
48. Thome, J.; Zhang, J.; Davids, E.; Foley, P.; Weijers, H. G.; Wiesbeck, G. A.; Boning, J.; Riederer, P.; Gerlach, M. Evidence for increased oxidative stress in alcohol-dependent patients provided by quantification of in vivo salicylate hydroxylation products. *Alcohol Clin. Exp. Res.* **1997**, *21*, 82–85.
49. Gibson, B. W. Exploiting proteomics in the discovery of drugs that target mitochondrial oxidative damage. *Sci. Aging Knowl. Environ.* **2004**, *11*, pe12.
50. Han, X. J.; Tomizawa, K.; Fujimura, A.; Ohmori, I.; Nishiki, T.; Matsushita, M.; Matsui, H. Regulation of mitochondrial dynamics and neurodegenerative diseases. *Acta Med. Okayama* **2011**, *65*, 1–10.
51. Vonsattel, J. P.; Keller, C.; Cortes Ramirez, E. P. Huntington's disease--Neuropathology. *Handb. Clin. Neurol.* **2011**, *100*, 83–100.
52. Gladden, J. D.; Ahmed, M. I.; Litovsky, S. H.; Schiros, C. G.; Lloyd, S. G.; Gupta, H.; Denney, T. S., Jr.; Darley-Usmar, V.; McGiffin, D. C.; Dell'italia, L. J. Oxidative stress and myocardial remodeling in chronic mitral regurgitation. *Am. J. Med. Sci.* **2011**, *342*, 114–119.
53. Oyanagi, E.; Yano, H.; Uchida, M.; Utsumi, K.; Sasaki, J. Protective action of L-carnitine on cardiac mitochondrial function and structure against fatty acid stress. *Biochem. Biophys. Res. Commun.* **2011**.
54. Kamp, D. W.; Shacter, E.; Weitzman, S. A. Chronic inflammation and cancer: The role of the mitochondria. *Oncology (Williston Park)* **2011**, *25*, 400–410, 413.
55. Dalle-Donne, I.; Scaloni, A.; Giustarini, D.; Cavarra, E.; Tell, G.; Lungarella, G.; Colombo, R.; Rossi, R.; Milzani, A. Proteins as biomarkers

of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom. Rev.* **2005**, *24*, 55–99.

56. Laragione, T.; Bonetto, V.; Casoni, F.; Massignan, T.; Bianchi, G.; Gianazza, E.; Ghezzi, P. Redox regulation of surface protein thiols: identification of integrin alpha-4 as a molecular target by using redox proteomics. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 14737–14741.
57. Fratelli, M.; Demol, H.; Puype, M.; Casagrande, S.; Eberini, I.; Salmona, M.; Bonetto, V.; Mengozzi, M.; Duffieux, F.; Miclet, E.; Bachi, A.; Vandekerckhove, J.; Gianazza, E.; Ghezzi, P. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 3505–3510.
58. Furukawa, A.; Kawamoto, Y.; Chiba, Y.; Takei, S.; Hasegawa-Ishii, S.; Kawamura, N.; Yoshikawa, K.; Hosokawa, M.; Oikawa, S.; Kato, M.; Shimada, A. Proteomic identification of hippocampal proteins vulnerable to oxidative stress in excitotoxin-induced acute neuronal injury. *Neurobiol. Dis.* **2011**, *43*, 706–714.
59. Song, B. J.; Suh, S. K.; Moon, K. H. A simple method to systematically study oxidatively modified proteins in biological samples and its applications. *Methods Enzymol.* **2010**, *473*, 251–264.
60. Brennan, J. P.; Wait, R.; Begum, S.; Bell, J. R.; Dunn, M. J.; Eaton, P. Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J. Biol. Chem.* **2004**, *279*, 41352–41360.
61. Leonard, S. E.; Carroll, K. S. Chemical ’omics’ approaches for understanding protein cysteine oxidation in biology. *Curr. Opin. Chem. Biol.* **2011**, *15*, 88–102.
62. Poon, H. F.; Castegna, A.; Farr, S. A.; Thongboonkerd, V.; Lynn, B. C.; Banks, W. A.; Morley, J. E.; Klein, J. B.; Butterfield, D. A. Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. *Neuroscience* **2004**, *126*, 915–926.
63. Bailey, S. M.; Landar, A.; Darley-Usmar, V. Mitochondrial proteomics in free radical research. *Free Radical Biol. Med.* **2005**, *38*, 175–188.
64. Ning, M.; Sarracino, D. A.; Kho, A. T.; Guo, S.; Lee, S. R.; Krastins, B.; Buonanno, F. S.; Vizcaino, J. A.; Orchard, S.; McMullin, D.; Wang, X.; Lo, E. H. Proteomic temporal profile of human brain endothelium after oxidative stress. *Stroke* **2011**, *42*, 37–43.
65. Morbt, N.; Tomm, J.; Feltens, R.; Mogel, I.; Kalkhof, S.; Murugesan, K.; Wirth, H.; Vogt, C.; Binder, H.; Lehmann, I.; von Bergen, M. Chlorinated benzenes cause concomitantly oxidative stress and induction of apoptotic markers in lung epithelial cells (A549) at nonacute toxic concentrations. *J. Proteome Res.* **2011**, *10*, 363–378.
66. Gill, R. R.; Gbur, C. J., Jr.; Fisher, B. J.; Hess, M. L.; Fowler, A. A., 3rd; Kukreja, R. C.; Sholley, M. M. Heat shock provides delayed protection against oxidative injury in cultured human umbilical vein endothelial cells. *J. Mol. Cell. Cardiol.* **1998**, *30*, 2739–2749.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

67. Jiang, B.; Xiao, W.; Shi, Y.; Liu, M.; Xiao, X. Heat shock pretreatment inhibited the release of Smac/DIABLO from mitochondria and apoptosis induced by hydrogen peroxide in cardiomyocytes and C2C12 myogenic cells. *Cell Stress Chaperones* **2005**, *10*, 252–262.

68. Mary, J.; Rogniaux, H.; Rees, J. F.; Zal, F. Response of *Alvinella pompejana* to variable oxygen stress: A proteomic approach. *Proteomics* **2010**, *10*, 2250–2258.

69. Jung, E. J.; Avliyakulov, N. K.; Boontheung, P.; Loo, J. A.; Nel, A. E. Pro-oxidative DEP chemicals induce heat shock proteins and an unfolding protein response in a bronchial epithelial cell line as determined by DIGE analysis. *Proteomics* **2007**, *7*, 3906–3918.

70. Rocha, M.; Esplugues, J. V.; Hernandez-Mijares, A.; Victor, V. M. Mitochondrial-targeted antioxidants and oxidative stress: A proteomic prospective study. *Curr. Pharm. Des.* **2009**, *15*, 3052–3062.

71. Victor, V. M.; Apostolova, N.; Herance, R.; Hernandez-Mijares, A.; Rocha, M. Oxidative stress and mitochondrial dysfunction in atherosclerosis: Mitochondria-targeted antioxidants as potential therapy. *Curr. Med. Chem.* **2009**, *16*, 4654–4667.

72. Victor, V. M.; Rocha, M.; Sola, E.; Banuls, C.; Garcia-Malpartida, K.; Hernandez-Mijares, A. Oxidative stress, endothelial dysfunction and atherosclerosis. *Curr. Pharm. Des.* **2009**, *15*, 2988–3002.

73. Chou, J. L.; Shenoy, D. V.; Thomas, N.; Choudhary, P. K.; Laferla, F. M.; Goodman, S. R.; Breen, G. A. Early dysregulation of the mitochondrial proteome in a mouse model of Alzheimer's disease. *J. Proteomics* **2011**, *74*, 466–479.

74. Perluigi, M.; Di Domenico, F.; Giorgi, A.; Schinina, M. E.; Coccia, R.; Cini, C.; Bellia, F.; Cambria, M. T.; Cornelius, C.; Butterfield, D. A.; Calabrese, V. Redox proteomics in aging rat brain: Involvement of mitochondrial reduced glutathione status and mitochondrial protein oxidation in the aging process. *J. Neurosci. Res.* **2010**, *88*, 3498–3507.

75. Rodolfo, C.; Ciccosanti, F.; Giacomo, G. D.; Piacentini, M.; Fimia, G. M. Proteomic analysis of mitochondrial dysfunction in neurodegenerative diseases. *Expert Rev. Proteomics* **2010**, *7*, 519–542.

76. Palmfeldt, J.; Vang, S.; Stenbroen, V.; Pavlou, E.; Baycheva, M.; Buchal, G.; Monavari, A. A.; Augoustides-Savvopoulou, P.; Mandel, H.; Gregersen, N. Proteomics reveals that redox regulation is disrupted in patients with ethylmalonic encephalopathy. *J. Proteome Res.* **2011**, *10*, 2389–2396.

77. Amici, A.; Levine, R. L.; Tsai, L.; Stadtman, E. R. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J. Biol. Chem.* **1989**, *264*, 3341–3346.

78. Green, B. N.; Suzuki, T.; Gotoh, T.; Kuchumov, A. R.; Vinogradov, S. N. Electrospray ionization mass spectrometric determination of the complete polypeptide chain composition of *Tylorrhynchus heterochaetus* hemoglobin. *J. Biol. Chem.* **1995**, *270*, 18209–18211.

79. Kim, J. S.; Raines, R. T. A misfolded but active dimer of bovine seminal ribonuclease. *Eur. J. Biochem.* **1994**, *224*, 109–114.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

80. Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **1990**, *186*, 464–478.
81. Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* **1994**, *233*, 346–357.
82. Lewisch, S. A.; Levine, R. L. Determination of 2-oxohistidine by amino acid analysis. *Anal. Biochem.* **1995**, *231*, 440–446.
83. Lewisch, S. A.; Levine, R. L. Determination of 2-oxohistidine by amino acid analysis. *Methods Enzymol.* **1999**, *300*, 120–124.
84. Requena, J. R.; Chao, C. C.; Levine, R. L.; Stadtman, E. R. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 69–74.
85. Requena, J. R.; Levine, R. L.; Stadtman, E. R. Recent advances in the analysis of oxidized proteins. *Amino Acids* **2003**, *25*, 221–226.
86. Ruoppolo, M.; Amoresano, A.; Pucci, P.; Pascarella, S.; Polticelli, F.; Trovato, M.; Menegatti, E.; Ascenzi, P. Characterization of five new low-molecular-mass trypsin inhibitors from white mustard (Sinapis alba L.) seed. *Eur. J. Biochem.* **2000**, *267*, 6486–6492.
87. Bienvenut, W. V.; Deon, C.; Pasquarello, C.; Campbell, J. M.; Sanchez, J. C.; Vestal, M. L.; Hochstrasser, D. F. Matrix-assisted laser desorption/ionization-tandem mass spectrometry with high resolution and sensitivity for identification and characterization of proteins. *Proteomics* **2002**, *2*, 868–876.
88. Perdivara, I.; Deterding, L. J.; Przybylski, M.; Tomer, K. B. Mass spectrometric identification of oxidative modifications of tryptophan residues in proteins: chemical artifact or post-translational modification? *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1114–1117.
89. Hartl, F. U. Molecular chaperones in cellular protein folding. *Nature* **1996**, *381*, 571–579.
90. Lindquist, S.; Craig, E. A. The heat-shock proteins. *Annu. Rev. Genet.* **1988**, *22*, 631–677.
91. Agashe, V. R.; Hartl, F. U. Roles of molecular chaperones in cytoplasmic protein folding. *Semin. Cell Dev. Biol.* **2000**, *11*, 15–25.
92. Arya, R.; Mallik, M.; Lakhotia, S. C. Heat shock genes - integrating cell survival and death. *J Biosci* **2007**, *32*, 595–610.
93. Soti, C.; Csermely, P. Aging cellular networks: chaperones as major participants. *Exp. Gerontol.* **2007**, *42*, 113–119.
94. Jolly, C.; Morimoto, R. I. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* **2000**, *92*, 1564–1572.
95. Takayama, S.; Reed, J. C.; Homma, S. Heat-shock proteins as regulators of apoptosis. *Oncogene* **2003**, *22*, 9041–9047.
96. Mosser, D. D.; Morimoto, R. I. Molecular chaperones and the stress of oncogenesis. *Oncogene* **2004**, *23*, 2907–2918.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

97. Garrido, C.; Brunet, M.; Didelot, C.; Zermati, Y.; Schmitt, E.; Kroemer, G. Heat shock proteins 27 and 70: Anti-apoptotic proteins with tumorigenic properties. *Cell Cycle* **2006**, *5*, 2592–2601.

98. Mosser, D. D.; Caron, A. W.; Bourget, L.; Meriin, A. B.; Sherman, M. Y.; Morimoto, R. I.; Massie, B. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol. Cell. Biol.* **2000**, *20*, 7146–7159.

99. Garrido, C.; Schmitt, E.; Cande, C.; Vahsen, N.; Parcellier, A.; Kroemer, G. HSP27 and HSP70: Potentially oncogenic apoptosis inhibitors. *Cell Cycle* **2003**, *2*, 579–584.

100. Bruey, J. M.; Ducasse, C.; Bonniaud, P.; Ravagnan, L.; Susin, S. A.; Diaz-Latoud, C.; Gurbuxani, S.; Arrigo, A. P.; Kroemer, G.; Solary, E.; Garrido, C. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat. Cell Biol.* **2000**, *2*, 645–652.

101. Paul, C.; Manero, F.; Gonin, S.; Kretz-Remy, C.; Virot, S.; Arrigo, A. P. Hsp27 as a negative regulator of cytochrome c release. *Mol. Cell. Biol.* **2002**, *22*, 816–834.

102. Chauhan, D.; Li, G.; Hideshima, T.; Podar, K.; Mitsiades, C.; Mitsiades, N.; Catley, L.; Tai, Y. T.; Hayashi, T.; Shringarpure, R.; Burger, R.; Munshi, N.; Ohtake, Y.; Saxena, S.; Anderson, K. C. Hsp27 inhibits release of mitochondrial protein Smac in multiple myeloma cells and confers dexamethasone resistance. *Blood* **2003**, *102*, 3379–3386.

103. Kampinga, H. H.; Hageman, J.; Vos, M. J.; Kubota, H.; Tanguay, R. M.; Bruford, E. A.; Cheetham, M. E.; Chen, B.; Hightower, L. E. Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* **2009**, *14*, 105–111.

104. Sreedhar, A. S.; Kalmar, E.; Csermely, P.; Shen, Y. F. Hsp90 isoforms: Functions, expression and clinical importance. *FEBS Lett.* **2004**, *562*, 11–15.

105. Pearl, L. H.; Prodromou, C. Structure and in vivo function of Hsp90. *Curr. Opin. Struct. Biol.* **2000**, *10*, 46–51.

106. Prodromou, C.; Panaretou, B.; Chohan, S.; Siligardi, G.; O'Brien, R.; Ladbury, J. E.; Roe, S. M.; Piper, P. W.; Pearl, L. H. The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. *EMBO J.* **2000**, *19*, 4383–4392.

107. Richter, K.; Soroka, J.; Skalniak, L.; Leskovar, A.; Hessling, M.; Reinstein, J.; Buchner, J. Conserved conformational changes in the ATPase cycle of human Hsp90. *J. Biol. Chem.* **2008**, *283*, 17757–17765.

108. Sidera, K.; Gaitanou, M.; Stellas, D.; Matsas, R.; Patsavoudi, E. A critical role for HSP90 in cancer cell invasion involves interaction with the extracellular domain of HER-2. *J. Biol. Chem.* **2008**, *283*, 2031–2041.

109. Cambiazo, V.; Gonzalez, M.; Isamit, C.; Maccioni, R. B. The beta-isoform of heat shock protein hsp-90 is structurally related with human microtubule-interacting protein Mip-90. *FEBS Lett.* **1999**, *457*, 343–347.

110. Eletto, D.; Dersh, D.; Argon, Y. GRP94 in ER quality control and stress responses. *Semin. Cell Dev. Biol.* **2010**, *21*, 479–485.

111. Lanneau, D.; Brunet, M.; Frisan, E.; Solary, E.; Fontenay, M.; Garrido, C. Heat shock proteins: Essential proteins for apoptosis regulation. *J. Cell. Mol. Med.* **2008**, *12*, 743–761.
112. Gething, M. J. Role and regulation of the ER chaperone BiP. *Semin. Cell. Dev. Biol.* **1999**, *10*, 465–472.
113. Daugaard, M.; Rohde, M.; Jaattela, M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett.* **2007**, *581*, 3702–3710.
114. Calabrese, V.; Cornelius, C.; Mancuso, C.; Pennisi, G.; Calafato, S.; Bellia, F.; Bates, T. E.; Giuffrida Stella, A. M.; Schapira, T.; Dinkova Kostova, A. T.; Rizzarelli, E. Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. *Neurochem. Res.* **2008**, *33*, 2444–2471.
115. Bukau, B.; Weissman, J.; Horwich, A. Molecular chaperones and protein quality control. *Cell* **2006**, *125*, 443–451.
116. Qiu, X. B.; Shao, Y. M.; Miao, S.; Wang, L. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cell. Mol. Life Sci.* **2006**, *63*, 2560–2570.
117. Cappello, F.; Conway de Macario, E.; Marasa, L.; Zummo, G.; Macario, A. J. Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy. *Cancer Biol. Ther.* **2008**, *7*, 801–809.
118. Quintana, F. J.; Cohen, I. R. The HSP60 immune system network. *Trends Immunol.* **2011**, *32*, 89–95.
119. Moudgil, K. D.; Durai, M. Regulation of autoimmune arthritis by self-heat-shock proteins. *Trends Immunol.* **2008**, *29*, 412–418.
120. Friedrich, K. L.; Giese, K. C.; Buan, N. R.; Vierling, E. Interactions between small heat shock protein subunits and substrate in small heat shock protein-substrate complexes. *J. Biol. Chem.* **2004**, *279*, 1080–1089.
121. Stromer, T.; Ehrnsperger, M.; Gaestel, M.; Buchner, J. Analysis of the interaction of small heat shock proteins with unfolding proteins. *J. Biol. Chem.* **2003**, *278*, 18015–18021.
122. Haslbeck, M.; Franzmann, T.; Weinfurtner, D.; Buchner, J. Some like it hot: The structure and function of small heat-shock proteins. *Nat. Struct. Mol. Biol.* **2005**, *12*, 842–846.
123. Buzzard, K. A.; Giaccia, A. J.; Killender, M.; Anderson, R. L. Heat shock protein 72 modulates pathways of stress-induced apoptosis. *J. Biol. Chem.* **1998**, *273*, 17147–17153.
124. Li, C. Y.; Lee, J. S.; Ko, Y. G.; Kim, J. I.; Seo, J. S. Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J. Biol. Chem.* **2000**, *275*, 25665–25671.
125. Ravagnan, L.; Gurbuxani, S.; Susin, S. A.; Maisse, C.; Daugas, E.; Zamzami, N.; Mak, T.; Jaattela, M.; Penninger, J. M.; Garrido, C.; Kroemer, G. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell. Biol.* **2001**, *3*, 839–843.
126. Lee, A. S.; Hendershot, L. M. ER stress and cancer. *Cancer Biol. Ther.* **2006**, *5*, 721–722.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

127. Hendershot, L. M. The ER function BiP is a master regulator of ER function. *Mt. Sinai J. Med.* **2004**, *71*, 289–297.

128. Shen, J.; Chen, X.; Hendershot, L.; Prywes, R. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev. Cell* **2002**, *3*, 99–111.

129. Creemers, J. W.; van de Loo, J. W.; Plets, E.; Hendershot, L. M.; Van De Ven, W. J. Binding of BiP to the processing enzyme lymphoma proprotein convertase prevents aggregation, but slows down maturation. *J. Biol. Chem.* **2000**, *275*, 38842–38847.

130. Bertolotti, A.; Zhang, Y.; Hendershot, L. M.; Harding, H. P.; Ron, D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* **2000**, *2*, 326–332.

131. Lee, Y. K.; Brewer, J. W.; Hellman, R.; Hendershot, L. M. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. *Mol. Biol. Cell* **1999**, *10*, 2209–2219.

132. Hellman, R.; Vanhove, M.; Lejeune, A.; Stevens, F. J.; Hendershot, L. M. The in vivo association of BiP with newly synthesized proteins is dependent on the rate and stability of folding and not simply on the presence of sequences that can bind to BiP. *J. Cell Biol.* **1999**, *144*, 21–30.

133. Hamman, B. D.; Hendershot, L. M.; Johnson, A. E. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. *Cell* **1998**, *92*, 747–758.

134. Lievremont, J. P.; Rizzuto, R.; Hendershot, L.; Meldolesi, J. BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. *J. Biol. Chem.* **1997**, *272*, 30873–30879.

135. Morris, J. A.; Dorner, A. J.; Edwards, C. A.; Hendershot, L. M.; Kaufman, R. J. Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J. Biol. Chem.* **1997**, *272*, 4327–4334.

136. Wei, J.; Gaut, J. R.; Hendershot, L. M. In vitro dissociation of BiP-peptide complexes requires a conformational change in BiP after ATP binding but does not require ATP hydrolysis. *J. Biol. Chem.* **1995**, *270*, 26677–26682.

137. Hendershot, L. M.; Valentine, V. A.; Lee, A. S.; Morris, S. W.; Shapiro, D. N. Localization of the gene encoding human BiP/GRP78, the endoplasmic reticulum cognate of the HSP70 family, to chromosome 9q34. *Genomics* **1994**, *20*, 281–284.

138. Platica, M.; Chen, H. Z.; Ciurea, D.; Gil, J.; Mandeli, J.; Hollander, V. P. Pituitary extract causes aggregation and differentiation of rat mammary tumor MTW9/Pl cells. *Endocrinology* **1992**, *131*, 2573–2580.

139. Platica, M.; Ivan, E.; Holland, J. F.; Ionescu, A.; Chen, S.; Mandeli, J.; Unger, P. D.; Platica, O. A pituitary gene encodes a protein that produces differentiation of breast and prostate cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 1560–1565.

140. Sokolowska, I.; Woods, A. G.; Gawinowicz, M. A.; Darie, C. C. Identification of tumor differentiation factor (TDF) receptor suggests a steroid-independent pathway. **2011**, under review.
141. Kang, B. H.; Plescia, J.; Dohi, T.; Rosa, J.; Doxsey, S. J.; Altieri, D. C. Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell* **2007**, *131*, 257–270.
142. Sawai, A.; Chandarlapaty, S.; Greulich, H.; Gonen, M.; Ye, Q.; Arteaga, C. L.; Sellers, W.; Rosen, N.; Solit, D. B. Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. *Cancer Res.* **2008**, *68*, 589–596.
143. Kamal, A.; Thao, L.; Sensintaffar, J.; Zhang, L.; Boehm, M. F.; Fritz, L. C.; Burrows, F. J. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* **2003**, *425*, 407–410.
144. Luk, J. M.; Lam, C. T.; Siu, A. F.; Lam, B. Y.; Ng, I. O.; Hu, M. Y.; Che, C. M.; Fan, S. T. Proteomic profiling of hepatocellular carcinoma in Chinese cohort reveals heat-shock proteins (Hsp27, Hsp70, GRP78) up-regulation and their associated prognostic values. *Proteomics* **2006**, *6*, 1049–1057.
145. Lim, S. O.; Park, S. G.; Yoo, J. H.; Park, Y. M.; Kim, H. J.; Jang, K. T.; Cho, J. W.; Yoo, B. C.; Jung, G. H.; Park, C. K. Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. *World J. Gastroenterol.* **2005**, *11*, 2072–2079.
146. Pandey, P.; Saleh, A.; Nakazawa, A.; Kumar, S.; Srinivasula, S. M.; Kumar, V.; Weichselbaum, R.; Nalin, C.; Alnemri, E. S.; Kufe, D.; Kharbanda, S. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J.* **2000**, *19*, 4310–4322.
147. Bando, Y.; Katayama, T.; Kasai, K.; Taniguchi, M.; Tamatani, M.; Tohyama, M. GRP94 (94 kDa glucose-regulated protein) suppresses ischemic neuronal cell death against ischemia/reperfusion injury. *Eur. J. Neurosci.* **2003**, *18*, 829–840.
148. Cowen, L. E.; Lindquist, S. Hsp90 potentiates the rapid evolution of new traits: Drug resistance in diverse fungi. *Science* **2005**, *309*, 2185–2189.
149. Cowen, L. E.; Singh, S. D.; Kohler, J. R.; Collins, C.; Zaas, A. K.; Schell, W. A.; Aziz, H.; Mylonakis, E.; Perfect, J. R.; Whitesell, L.; Lindquist, S. Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 2818–2823.
150. Henderson, B.; Pockley, A. G. Molecular chaperones and protein-folding catalysts as intercellular signaling regulators in immunity and inflammation. *J. Leukocyte Biol.* **2010**, *88*, 445–462.
151. Pockley, A. G.; Muthana, M.; Calderwood, S. K. The dual immunoregulatory roles of stress proteins. *Trends Biochem. Sci.* **2008**, *33*, 71–79.
152. Powers, M. V.; Workman, P. Inhibitors of the heat shock response: Biology and pharmacology. *FEBS Lett.* **2007**, *581*, 3758–3769.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

153. Schmitt, E.; Gehrmann, M.; Brunet, M.; Multhoff, G.; Garrido, C. Intracellular and extracellular functions of heat shock proteins: Repercussions in cancer therapy. *J. Leukocyte Biol.* **2007**, *81*, 15–27.
154. Lanneau, D.; de Thonel, A.; Maurel, S.; Didelot, C.; Garrido, C. Apoptosis versus cell differentiation: Role of heat shock proteins HSP90, HSP70 and HSP27. *Prion* **2007**, *1*, 53–60.
155. Zhang, Y.; Liu, R.; Ni, M.; Gill, P.; Lee, A. S. Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. *J. Biol. Chem.* **2010**, *285*, 15065–15075.
156. Mambula, S. S.; Calderwood, S. K. Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. *J. Immunol.* **2006**, *177*, 7849–7857.
157. Ni, M.; Zhang, Y.; Lee, A. S. Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. *Biochem. J.* **2011**, *434*, 181–188.
158. Binder, R. J.; Vatner, R.; Srivastava, P. The heat-shock protein receptors: Some answers and more questions. *Tissue Antigens* **2004**, *64*, 442–451.
159. Palotai, R.; Szalay, M. S.; Csermely, P. Chaperones as integrators of cellular networks: Changes of cellular integrity in stress and diseases. *IUBMB Life* **2008**, *60*, 10–18.
160. Arrigo, A. P.; Firdaus, W. J.; Mellier, G.; Moulin, M.; Paul, C.; Diaz-latoud, C.; Kretz-remy, C. Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression. *Methods* **2005**, *35*, 126–138.
161. Read, D. E.; Gorman, A. M. Heat shock protein 27 in neuronal survival and neurite outgrowth. *Biochem. Biophys. Res. Commun.* **2009**, *382*, 6–8.
162. Rogalla, T.; Ehrnsperger, M.; Preville, X.; Kotlyarov, A.; Lutsch, G.; Ducasse, C.; Paul, C.; Wieske, M.; Arrigo, A. P.; Buchner, J.; Gaestel, M. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J. Biol. Chem.* **1999**, *274*, 18947–18956.
163. Wyttenbach, A.; Sauvageot, O.; Carmichael, J.; Diaz-Latoud, C.; Arrigo, A. P.; Rubinsztein, D. C. Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* **2002**, *11*, 1137–1151.
164. Campanella, C.; Bucchieri, F.; Ardizzone, N. M.; Marino Gammazza, A.; Montalbano, A.; Ribbene, A.; Di Felice, V.; Bellafiore, M.; David, S.; Rappa, F.; Marasa, M.; Peri, G.; Farina, F.; Czarnecka, A. M.; Conway de Macario, E.; Macario, A. J.; Zummo, G.; Cappello, F. Upon oxidative stress, the antiapoptotic Hsp60/procaspase-3 complex persists in mucoepidermoid carcinoma cells. *Eur. J. Histochem.* **2008**, *52*, 221–228.
165. Timperio, A. M.; Egidi, M. G.; Zolla, L. Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP). *J. Proteomics* **2008**, *71*, 391–411.
166. Huang, B.; Xu, C. Identification and characterization of proteins associated with plant tolerance to heat stress. *J. Integr. Plant Biol.* **2008**, *50*, 1230–1237.

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

Downloaded by DUKE UNIV on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ch013

167. Li, J. Y.; Yang, H. J.; Lan, T. Y.; Wei, H.; Zhang, H. R.; Chen, M.; Fan, W.; Ma, Y. Y.; Zhong, B. X. Expression profiling and regulation of genes related to silkworm posterior silk gland development and fibroin synthesis. *J. Proteome Res.* **2011**.

168. Jia, M.; Souchelnytskyi, S. Proteome profiling of heat shock of human primary breast epithelial cells, a dataset report. *Cell Stress Chaperones* **2011**, *16*, 459–467.

169. Darie, C. C.; Deinhardt, K.; Zhang, G.; Cardasis, H. S.; Chao, M. V.; Neubert, T. A. Identifying transient protein-protein interactions in EphB2 signaling by Blue Native PAGE and mass spectrometry. *Proteomics* **2011**.

170. McManus, C. A.; Donoghue, P. M.; Dunn, M. J. A fluorescent codetection system for immunoblotting and proteomics through ECL-Plex and CyDye labeling. *Methods Mol. Biol.* **2009**, *536*, 515–526.

171. Han, C.; Park, I.; Lee, B.; Jin, S.; Choi, H.; Kwon, J. T.; Kwon, Y. I.; Kim do, H.; Park, Z. Y.; Cho, C. Identification of heat shock protein 5, calnexin and integral membrane protein 2B as Adam7-interacting membrane proteins in mouse sperm. *J. Cell Physiol.* **2011**, *226*, 1186–1195.

172. Koo, J.; Kim, K. I.; Min, B. H.; Lee, G. M. Differential protein expression in human articular chondrocytes expanded in serum-free media of different medium osmolalities by DIGE. *J. Proteome Res.* **2010**, *9*, 2480–2487.

173. DeAngelis, J. T.; Li, Y.; Mitchell, N.; Wilson, L.; Kim, H.; Tollefsbol, T. O. 2D difference gel electrophoresis analysis of different time points during the course of neoplastic transformation of human mammary epithelial cells. *J. Proteome Res.* **2011**, *10*, 447–458.

174. Ioannidis, J. P. A roadmap for successful applications of clinical proteomics. *Proteomics Clin. Appl.* **2011**, *5*, 241–247.

Downloaded by STANFORD UNIV GREEN LIBR on June 4, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ot001

Editors' Biographies

Silvana Andreescu

Silvana Andreescu is an Associate Professor in the Department of Chemistry and Biomolecular Science at Clarkson University, Potsdam, NY. She received a joint Ph.D. degree in chemistry from the University of Perpignan, France, and University of Bucharest, Romania. Her research expertise is in analytical and bioanalytical chemistry focusing on the development, characterization and application of chemical and biological sensors for clinical, food and environmental monitoring. She has published more than 55 peer-reviewed journal articles and 11 book chapters and has delivered some 100 presentations at professional and academic conferences. Recent work includes investigations of basic biochemical mechanisms at bio-interfaces, biomimetic materials with antioxidant properties for diagnosis and therapeutic applications, toxicity of engineered nanomaterials and development of electrochemical probes for study of neurological activity, nanoparticle-induced toxicity and oxidative stress. She is the recipient of a French Government Graduate Fellowship, a NATO-NSF Postdoctoral Fellowship and the NSF-CAREER award.

Maria Hepel

Maria Hepel received the M.S. and Ph.D. degrees in chemistry from Jagellonian University in Krakow, Poland. From 1985 she worked as the Faculty at the State University of New York at Potsdam where she is now a Professor and Chair of the Department of Chemistry. She has published over 140 papers, 25 chapters in books, and has made over 400 presentations at the national and international symposia. She organized many symposia at the American Chemical Society, Electrochemical Society and International Society of Electrochemistry Meetings and has been the program chair of the 2010 North-East Regional Meeting of ACS. Her current research interests include DNA intercalation sensors, piezoimmunosensors, sensors for biomarkers of oxidative stress and cancer, fluorescence energy transfer (FRET and NSET), DNA-hybridization biosensors, microsensor arrays and lab-on-a-chip systems, photovoltaics, dye pollutant degradation, and electrochromic devices. She won the SUNY Potsdam President's Award for Excellence in Research and Creative Endeavor in 1995 and 2001, the SUNY Chancellor's Award for Excellence in Teaching in 1998 and SUNY Chancellor's Award for Research in 2003.

In Oxidative Stress: Diagnostics, Prevention, and Therapy; Andreescu, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Subject Index

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

A

- Acetyl-L-carnitine, 60
- Adenosine triphosphate (ATP), 1, 50
- Aerobic exercise, 62
- AFMK. *See* N^1-Acetyl-N^2-formyl-5-methoxykynuramine (AFMK)
- Agarose gel electrophoresis, 181
- Ageing, 28
- *Aggregatibacter actinomycetemcomitans*, 301
- Alkaline elution technique, 212
- ALS. *See* Amyotrophic lateral sclerosis (ALS)
- Alzheimer's disease (AD), 22, 106, 260
  - oxidative stress, 260
- American Cancer Society, 106
- American Heart Association, 106
- Amino acids
  - oxidation, 387
  - oxidative damages, 384
  - product modifications, 386*t*
  - proteomics experiment, 387
- Aminophenylfluorescein (APF), 319
- Amyotrophic lateral sclerosis (ALS), 262
  - oxidative stress, 263
- 4-Androstene-3,17-dione, 85
- Anthocyanins, 11
- Antioxidant enzymes, 45
- Antioxidant response elements (ARE), 22
- Antioxidants
  - concentration dependence of ctDNA, 189*f*
  - effects, 94*f*
  - evaluation of capacity, 243
  - functionalized nanoparticles, 242
  - human health
    - classification, 6*t*
    - defined, 4
    - against disease, 18
    - effects, 29*t*
    - foods consumption, 10
    - health, 10
    - management of oxidative stress, 7*f*
    - perspectives and future directions, 33
    - phyto-constituents, 16
    - prophylaxis, 18
    - risks, 32
    - supplements, 10
    - synthetic, 17
    - therapy, 18
    - traditional medicinal systems, 12
- Anti-phosphotyrosine (pY99-IP), 351
- APF. *See* Aminophenylfluorescein (APF)
- ARE. *See* Antioxidant response elements (ARE)
- Artificial nanoparticle antioxidants
  - biodistribution, 244
  - classes, 237
  - evaluation of capacity, 243
  - therapy and disease prevention, 246
  - toxicity, 244
- Ascorbic acid, 24
- ATP. *See* Adenosine triphosphate (ATP)

B

- Bacteria transformation, 181
- BAS. *See* Bioanalytical systems (BAS)
- BBB. *See* Blood brain barrier (BBB)
- β-Carotene, 11
- BDNF. *See* Brain-derived neurotrophic factor (BDNF)
- BHA. *See* Butylated hydroxyanisole (BHA)
- BHT. *See* Butylated hydroxytoluene (BHT)
- Bioanalytical systems (BAS), 180
- Biochemical fractionation, 372
- Biosensors
  - hydrogen peroxide, 298, 299*t*
  - superoxide, 291, 292*t*
- BioWhittaker Molecular Applications (BMA), 181
- Blood brain barrier (BBB), 22, 261
- Blue Native polyacrylamide gel electrophoresis (BN-PAGE), 341
  - analysis of sub-complex 1 of the eukaryotic initiation factor 3 hetero-protein complex, 350*f*
  - experiments, 343
  - homo-protein complex, analysis, 349*f*
  - identification of transient protein-protein interactions in primary neurons, 353*f*
  - investigating mitochondrial protein complexes, 343
  - limitations, 359
  - mass spectrometry as methods for examining oxidative stress, 358
  - against oxidative stress, 354
  - principles, similarities and differences, 344*f*

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

  subunit composition of stable homo- and hetero-protein complexes, 348
  tool for determination of the Mw of protein complexes, 345
  tool in analysis of protein self-assembly into higher Mw polymers, 353
  WB as tool for identification of transient protein-protein interactions, 351
BMA. *See* BioWhittaker Molecular Applications (BMA)
BN-PAGE. *See* Blue Native polyacrylamide gel electrophoresis (BN-PAGE)
Boltzmann function, 186
Brain-derived neurotrophic factor (BDNF), 351
Breast cancer, 90
Bromophenol blue (BPB), 181
Butylated hydroxyanisole (BHA), 17
Butylated hydroxytoluene (BHT), 17

C

Cancer, 19
  prevention by antioxidant compound, 92
Carbohydrates, 9
Carbon atom, 8
Carbon Fiber Electrodes (CFE), 325
Carcinogenicity, estrogens, 89
Cardiomyopathy (CM), 52
Cardiovascular disease (CVD), 18, 111
CAT. *See* Catalase (CAT)
Catalase (CAT), 5, 113
Catalase-loaded magnetic nanoparticles, 243
Catechins, 11
Catechol, 178
  bacteria transformation, 181
  chemicals, 214
  electrochemical measurements, 215
  electrochemical reactions, 179
  fluorimetric measurements, 180
  gel electrophoresis, 181
  mediated oxidative DNA damage, 199
  preparation of damaged DNA samples, 181
  pro-oxidant properties, 179
  quantum mechanical calculations, 182
4-Catechol estrogen, 89
Catechol-*O*-methytransferase (COMT), 85
CD8 cells, 41
CD38 molecule, 41
Cell redox environment, 77
Cellular effects, 115
Cellular tropism, 40
Central nervous system (CNS), 41, 46, 261
Cerebral ischemia, 257
  sources of ROS during, 258*f*
Ceria nanoparticles
  heterogeneous catalysis, 240
  reduce intracellular ROS, 239*f*
Cerium oxide nanoparticles
  comparison of four recent studies, 271*t*
  efficacy of some selected antioxidants, 265*t*
  next generation antioxidant therapy for neurodegenerative diseases, 263
  oxidative stress biology, 255
CFE. *See* Carbon Fiber Electrodes (CFE)
Chalcone isomerase (CHI), 101
Chalcone synthase (CHS), 101
Chemiluminescent probes, 305
CHI. *See* Chalcone isomerase (CHI)
Christian amatore, 294
Chronic obstructive pulmonary disease (COPD), 24
Chronic renal failure, 24
CHS. *See* Chalcone synthase (CHS)
CK-MB. *See* Creatine kinase MB (CK-MB)
4CL. *See* 4-Coumaroyl–CoA ligase (4CL)
CM. *See* Cardiomyopathy (CM)
CN-PAGE. *See* Colorless Native PAGE (CN-PAGE)
CNS. *See* Central nervous system (CNS)
Colorless Native PAGE (CN-PAGE), 342
  pre-purification tool, 356
  principles, similarities and differences, 344*f*
  stable protein complex protective against oxidative stress in BN-PAGE, 356
COMT. *See* Catechol-*O*-methytransferase (COMT)
Coomassie dye, 343, 348
COPD. *See* Chronic obstructive pulmonary disease (COPD)
4-Coumaroyl–CoA ligase (4CL), 101
Creatine kinase MB (CK-MB), 113
CVD. *See* Cardiovascular disease (CVD)
Cyclic voltammetry, 327
Cyclic voltammograms, 218
Cysteine residues, oxidation, 315
Cytochrome c, 295
  electrochemical detection of the superoxide, 296*f*
Cytochrome P450, 4

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

D

DAHP. *See* 2-keto-3-deoxyarabina-heptulosonate- 7-phosphate (DAHP)
DAPI. *See* 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)
Deletion mutants, 51
Density functional theory (DFT), 182, 216
DES. *See* Diethylstilbestrol (DES)
DFT. *See* Density functional theory (DFT)
Diabetes mellitus, 20
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), 214, 217*f*
  analytes and probes, 217*f*
  dependence on temperature, 186*f*, 223*f*
  effect of GSH on temperature dependence, 187*f*
  fluorescence emission spectra, 184*f*
  fluorescence on temperature after interactions of ctDNA, 223*f*, 224*f*
Diamond nanoparticles, 238*f*
Di-cysteine proteins, 77
Dietary antioxidant, 10
Dietary factors, 3
Diethylstilbestrol (DES), 84
Differential Gel Electrophoresis (DIGE), 359
Differential pulse amperometry, 294
Differential pulse voltammograms (DPV), 180, 192*f*
  dependence, 193*f*
DIGE. *See* Differential Gel Electrophoresis (DIGE)
Dihydrolipoic acid, 93
5,5-Dimethyl-1-pyrroline N-oxide (DMPO), 79
2,2-Diphenyl-1-picrylhydrazyl (DPPH), 119
Direct infusion, ESI-MS and ESI-MS/MS, 378
DMPO. *See* 5,5-Dimethyl-1-pyrroline N-oxide (DMPO)
DNA, 9
DNA biosensors, gold electrodes, 215
DNA damage, 181
  catechol/Cu^{2+}-mediated, 187
  dependence of NB current, 221*f*
  detection, 182
  fluorimetric measurements, 221
  measuring changes in DNA melting-onset temperature, 222
  mechanism of GSH protection, 198
  monitoring melting characteristics, 182
  oxidative stress, toxicants and alkylating agents, 183*s*
  PQ induced, 225
  using electrochemical biosensors, 216
  voltammetric monitoring, 190
DNA oxidation, 178
DPPH. *See* 2,2-Diphenyl-1-picrylhydrazyl (DPPH)
DPV. *See* Differential pulse voltammograms (DPV)
Dulbecco's Modified Eagle's Medium, 304

E

EAE. *See* Experimental autoimmune encephalomyelitis (EAE)
EDC. *See* N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC)
EDOT. *See* 3,4-Ethylenedioxythiophene (EDOT)
EGCG. *See* Epigallocatechin-3-gallate (EGCG)
EGFR. *See* Epidermal growth factor receptor (EGFR)
eIF3. *See* Eukaryotic translation initiation factor 3 complex (eIF3)
Electrochemical sensor array chip (ESA), 322
Electrochemical sensors
  hydrogen peroxide, 298
  superoxide, 291, 292*t*, 293*f*
Electromotive force, 74
Electron paramagnetic resonance (EPR), 2, 79, 245
Electron Spin Resonance (ESR) spectroscopy, 79
Electrospray ionization, 370
Endogenous scavengers, 315
Endothelial dysfunction, 18
Enzyme regulation mechanisms, 121
E-4-P. *See* Erytheose-4-phosphate (E-4-P)
Eph receptors, 346
Ephrin signaling, workflow for the biochemical and MS characterization, 347*f*
Epidermal growth factor receptor (EGFR), 147
Epigallocatechin-3-gallate (EGCG), 102
EPR. *See* Electron paramagnetic resonance (EPR)
ERKO/*wnt-1* mice, 89
Erytheose-4-phosphate (E-4-P), 101
ESA. *See* Electrochemical sensor array chip (ESA)
Escherichia coli
  oxidative stress and antioxidant defenses, 125

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

  - possible mechanisms of ROS generation, 126*f*
- ESI-Q-TOF MS, 376, 377*f*
- ESR. *See* Electron Spin Resonance (ESR) spectroscopy
- Estrogens
  - carcinogenicity, 89
  - DNA adducts, 87
    - people with and without cancer, 90, 91*f*
  - formation, metabolism and DNA adducts, 86*f*
  - metabolic activation to form depurinating DNA adducts, 85*f*
  - metabolic pathway in cancer initiation, 84*f*
  - metabolism, 85
    - imbalances, 89
  - mutagenicity, 87
  - oxidative metabolism of, 83
  - semiquinones, 86
  - transformation of cells, 88
- 3,4-Ethylenedioxythiophene (EDOT), 326
- Eukaryotic translation initiation factor 3 complex (eIF3), 350
- Experimental autoimmune encephalomyelitis (EAE), 261
- Eye disorders, 25

F

- Faraday constant, 74
- Fas/APO-1/CD95 receptors, 42
- Fatal hepatomegaly, 52
- Fenton cascade, 177
  - defined, 182
- Ferric reducing ability assay (FRAP), 119
- Flavonoids, 11, 48, 101
  - basic structures, 104*f*
  - biosynthetic pathways in plants, 105*f*
  - subclasses, 103*t*
- Flavoprotein enzyme, 5
- FRAP. *See* Ferric reducing ability assay (FRAP)
- Free radical reactions, thermodynamics, 72
- Free radicals, 2
  - biological role, 4
  - biology, concept, 2
  - scavenging, 4
  - sources, 3
  - steady state, 4

G

- Gastrointestinal diseases, 23
- Gastrointestinal tract (GIT), 23
- GCE. *See* Glassy carbon electrode (GCE)
- Gel electrophoresis, 181
  - control experiments, 197, 197*f*
- Gershman's free radical theory, 2
- Gibbs energy, 73
- *Ginkgo biloba*, 22
- GIT. *See* Gastrointestinal tract (GIT)
- Glassy carbon electrode (GCE), 180
- Glucose transporter 1 (Glut1), 113
- Glut1. *See* Glucose transporter 1 (Glut1)
- Glutamate excitotoxicity, 257
- Glutathione
  - cell compartments, 78
  - effect of ROS, 78
  - master antioxidant, 78
- Glutathione (GSH), 72
- Glutathione disulphide (GSSG), 5
- Glutathione intervention, overview, 177
- Glutathione peroxidase (GP_x), 5
- Glutathione peroxidase (GSHPx), 148
- Glutathione reductase (GR), 5
- Glutathione S-transferase (GST), 62
- Glutathione thiyl radical, electronic structure, 196*f*
- Glycolisis, 50
- Gold electrodes
  - DNA biosensors, 215
  - scheme of the modification, 218*f*
- Gold nanoparticles, 246
- Golgi apparatus, 348
- GP_x. *See* Glutathione peroxidase (GP_x)
- GR. *See* Glutathione reductase (GR)
- Green synthesis, 245
- GRP78 proteins
  - expression, 393
  - IF detection, 396*f*
- GSH. *See* Glutathione (GSH)
- GSHPx. *See* Glutathione peroxidase (GSHPx)
- GSSG. *See* Glutathione disulphide (GSSG)
- GST. *See* Glutathione S-transferase (GST)

H

- HAART. *See* Highly active antiretroviral therapy (HAART)
- Half-cell reduction potentials, 72
  - selected couples, 73*t*
- Hartree-Fock methods, 182

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

HDF-a. *See* Human dermal fibroblasts (HDF-a)
Heat Shock Factor (HSF), 390
Heat Shock Protein (HSP), 383, 388
  characterization, 390
  expression, 393
  functions, 390
Hemin-only electrodes, 326
  FESEM images, 327*f*
  surface morphology, 326
Hemin-PEDOT film
  amperometric microsensor, 329, 330*f*
  chronoamperometric responses, 329*f*
  microsensor, 330, 331*f*
HIF1-α. *See* Hypoxia-inducible factor 1-α (HIF1-α)
Highly active antiretroviral therapy (HAART), 41, 49
  biomolecule oxidative damage and antioxidant deficiency in HIV/aids patients, 54*t*
HIV. *See* Human Immunodeficiency Virus (HIV)
Horseradish peroxidase, 302
  electrochemical detection of the hydrogen peroxide, 300*f*
HPF. *See* Hydroxyphenylfluorescein (HPF)
HSF. *See* Heat Shock Factor (HSF)
HSP. *See* Heat Shock Protein (HSP)
HSP60 proteins, 391
  roles, 393
HSP70 proteins, 391
  expression, 393
  IF detection, 396*f*
  roles, 392
HSP90 proteins, 390
  roles, 393
Human cells
  oxidative stress and antioxidant defenses, 138
  polyphenol antioxidant activity, 139
  polyphenol enzyme regulation, 146
  polyphenol ROS and RNS scavenging mechanisms, 143
  polyphenol-metal chelation, 148
  ROS/RNS- and metal-ion-induced signaling pathways, 140*f*
Human dermal fibroblasts (HDF-a), 394
Human Immunodeficiency Virus (HIV), 40
  altered redox indexes, 42
  antioxidant supplementation, 56
    control studies evidences of nutritional intervention, 57*t*
  apoptosis, 46
  biology and tropism, 40
  disease evolution and management, 41
  evidences of biomolecule oxidative damage and antioxidant deficiency, 44*t*
  HAART, 49
  hallmark of HIV infection, 40
  infected populations, 43
  infection, 47
  infects cells, 40
  micronutrients deficiency, 47
  nutritional requirements, 47
  nutritional requirements index, 40
  physical exercise, 61
  redox regulation, 49
  replication and oxidative stress, 45
  Se-deficient nutritional conditions, 46
Human umbilical vein endothelial cells (HUVEC), 114, 147
HUVEC. *See* Human umbilical vein endothelial cells (HUVEC)
Hydrogen peroxide
  analytical tools, 291
  biosensors, 298, 299*t*
  cellular level, 304
  direct electrochemical detection, 298
  electrochemical sensors, 291, 298
  half-life, 290
  living cell detection, 290
  mediated electrochemical detection, 301
Hydrogen peroxide regulation, 127
8-Hydroxydeoxyguanosine (8-OHdG), 9
6-Hydroxydopamine (6-OHDA), 146, 259
Hydroxyl radical, 86, 138
  formation, 228
  oxidation, 51
Hydroxyphenylfluorescein (HPF), 319
Hyperglycemia, 20
Hypoxia-inducible factor 1-a (HIF1-a), 113
Hypoxia-reoxygenation injury, 113

I

ICAM1. *See* Intercellular adhesion molecule 1 (ICAM1)
Immune system, 3
Individual Nutrient Intake Level (INL98) levels, 48
Infertility, 26
Inflammation, 3
Inorganic nanoparticles
  functionalized and composite, 242
  with intrinsic antioxidant properties as free radical scavengers, 237
Intercellular adhesion molecule 1 (ICAM1), 113

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

Internal ribosome entry site (IRES), 46
IRES. *See* Internal ribosome entry site (IRES)
IRES trans-acting factor (ITAF), 46
Iron regulatory protein (IRP), 317
IRP. *See* Iron regulatory protein (IRP)
Ischemic cascade, schematic diagram, 257*f*
ITAF. *See* IRES trans-acting factor (ITAF)

K

2-Keto-3-deoxyarabina-heptulosonate-7-phosphate (DAHP), 101

L

Lactate dehydrogenase (LDH), 113
L-Carnitine antioxidants, 214
  PQ-induced DNA damage, 225
LC-MS/MS, 379
  disulfide bridges within Vitelline envelope protein beta, 389*f*
  experiment, 380*f*
  oxidation of methionine, 385*f*
  peptide mixture, identification of HSP70 family member (GRP78), 395*f*
LDH. *See* Lactate dehydrogenase (LDH)
L-Dopa. *See* Levodopa (L-dopa)
Levodopa (L-dopa), 259
Lipatrophy, 55
Lipid hydroperoxide, reduction potential, 144*t*
Lipid peroxidation, 3, 8
Lipid peroxyl radical, 8
Lipids, 8
Lipodystrophic syndrome, 52
Lipoic acid, 5
Long terminal repeat (LTR), 40
LTR. *See* Long terminal repeat (LTR)
Lycopene, 11
Lymphocytes, 41

M

MACS. *See* Multicenter AIDS Cohort Study (MACS)
Major groove nucleobases, 194
MALDI. *See* Matrix Assisted Laser Desorption Ionization (MALDI)
Manganese superoxide dismutase, 314
Mangifera indica, 59
Mass spectrometry (MS), 373
  data analysis, 382
  ionization techniques, 373
  oxidative stress, 382
  proteomic analysis, 370
Matrix Assisted Laser Desorption Ionization (MALDI), 370
  TOF MS, 374, 375*f*
MCE. *See* Microchip electrophoresis (MCE)
MCF-7 cancer cells, 297
MD. *See* Molecular dynamics (MD)
Melatonin, 93
Melatonin antioxidants
  electronic structures, 228*f*
  PQ-induced DNA damage, 225
Mercaptopropionic acid (MPA), 214, 217
Metabolic process, 3
Metal chelation mechanisms, 122
Metal electrodes, electrochemical detection of the hydrogen peroxide, 300*f*
Methylamine irisolidone, 113
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 123, 259
Methyl viologen, 214
Microchip electrophoresis (MCE), 322
Micronutrients deficiency, 40
  HIV, 47
Mini-Gel Horizontal Electrophoresis System, 181
Mitochondria
  dysfunction hypothesis during antiviral treatment, 50
  dysfunctions, 21
  essential elements, 50
  toxicity, 49
Molecular damage, 8
Molecular dynamics (MD), 194
  simulation, 194
    interaction of PQ with dsDNA, 226, 227*f*
Moricandia arvensis, 131
MPA. *See* Mercaptopropionic acid (MPA)
MPGly. *See* N-(2-Mercapto-propionyl)glycine (MPGly)
MPTP. *See* 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)
MS. *See* Mass spectrometry (MS); Multiple sclerosis (MS)
Multicenter AIDS Cohort Study (MACS), 58
Multiple sclerosis (MS), 261
  oxidative stress, 261

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

N

NAC. *See* *N*-Acetyl cysteine (NAC)
NAcCys. *See* *N*-Acetylcysteine (NAcCys)
N-Acetyl cysteine (NAC), 20, 92
  effects, 95*f*
N-Acetylcysteine (NAcCys), 56, 188
  effect of GSH precursor, 188*f*
N^1-Acetyl-N^2-formyl-5-methoxykynuramine (AFMK), 213
Nanocarrier, 243
  magnetically responsive antioxidant, 243
Nanoceria, 264
  ability, 267
  free radical scavenging capabilities, 269
  industrial applications, 267
  model of the reaction mechanism for the oxidation of hydrogen peroxide, 267*f*
  reaction mechanism for the complete dismutation of hydrogen peroxide, 268*f*
Nanoencapsulated antioxidants, 242
NB. *See* Nile Blue (NB)
Ndh complex
  BN-PAGE PAGE 2D and its characterization by MALDI MS, 357*f*
  structural characterization, 355*f*
N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), 215
Necrotic lesions, 212
Nernst equation, 74
  half cell reduction potential, 76
N-Ethylmaleimide, 292
Neurodegenerative diseases, 21
Neuroglobin (Nb), 113
Neuronal biochemical composition, 21
NFkB. *See* Nuclear factor kB (NFkB)
Nicotinamide adenine dinucleotide system, 75
Nile Blue (NB), 214
  fluorescence emission spectra, 222*f*
NI-NOR. *See* Nitrite/nitric oxide-oxidoreductase (NI-NOR)
Nitrate reductase (NR), 317
Nitric oxide synthase (NOS), 20, 257, 312
Nitrite/nitric oxide-oxidoreductase (NI-NOR), 317
Nitrosoperoxycarbonate adduct, 312
3-Nitrotyrosine (3-NT), 257
N-(2-Mercapto-propionyl)glycine (MPGly), 189
  effect, 190*f*
Nonprotein thiol compound (NPSH), 5
Non-transferrin-bound iron (NTBI), 150
NOS. *See* Nitric oxide synthase (NOS)
NPSH. *See* Nonprotein thiol compound (NPSH)
NR. *See* Nitrate reductase (NR)
Nrf2. *See* Nuclear factor-E2-related factor (Nrf2)
3-NT. *See* 3-Nitrotyrosine (3-NT)
NTBI. *See* Non-transferrin-bound iron (NTBI)
Nuclear factor kB (NFkB), 23
Nuclear factor-E2-related factor (Nrf2), 22
Nutrient antioxidant, 10
NXY-059, 263

O

6-OHDA. *See* 6-Hydroxydopamine (6-OHDA)
8-OHdG. *See* 8-Hydroxydeoxyguanosine (8-OHdG)
ORAC. *See* Oxygen radical absorbance capacity (ORAC)
Ortho-quinones, 84
OS. *See* Oxidative stress (OS)
12-O-Tetradecanoylphorbol-13-acetate (TPA), 146
Oxidation, 72
Oxidative damages, amino acids, 384
Oxidative stress biology, 255
Oxidative stress (OS), 8, 40
  analysis of redox systems, 388
  antioxidant defenses in escherichia coli, 125
  antioxidant defenses in human cells, 138
  clinical evidences of NRTI mitochondrial toxicity, 52
  DNA damage, 115
  HIV infection and NRTI treatment, 51
  major cellular sources, 116*f*
  mitochondria and mutations of mtDNA in NRTI toxicity, 51
  use of proteomics to investigate, 383
OxoGua, 191
Oxygen
  properties, 1
  toxic properties, 2
Oxygen radical absorbance capacity (ORAC), 243
OxyR regulon, 127
  E. coli, 128*f*
  proteins regulated, 127*t*

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

P

PAL. *See* Phenylalanine ammonia lyase (PAL)
Paraquat-mediated DNA damage, 212
  electrochemical measurements, 215
  fluorimetric measurements, 216
  materials and methods, 214
  molecular dynamic simulations, 216
  preparation of DNA biosensors, 215
  quantum mechanical calculations, 216
Parkinson's disease (PD), 106, 112, 212, 258
  oxidative stress, 259
Particle size, 240
PBMC. *See* Peripheral blood mononuclear cells (PBMC)
PD. *See* Parkinson's diseases (PD)
PEG. *See* Polyethylene glycol (PEG)
Peripheral blood mononuclear cells (PBMC), 53
Peroxides, 43
Peroxisomes, 255
Peroxynitrite (PON)
  analytical challenges in face of biological complexity, 318
  analytical methods and techniques for detecting, 320
  biochemical mechanisms inducing toxicity in animals, 314
  chemistry, 312
  electrochemical methods, 321
  endogenous scavengers, 315
  exogenous scavengers, 316
  formation, 325*f*
  formation in plant organisms, 316
  formula, 312
  long-illusive biological pathway, 315
  major pathways, 313*f*
  non-electrochemical techniques, 320
  PEDOT/hemin nanostructured catalytic film, 324
  plant physiology and hypersensitive defense response, 317
  plant response to pathogens, 316
  research articles bearing, 332*f*
  synthesis, 324
  therapeutic approaches to mitigate the toxic effects, 315
Phagocytes, 290
PhenO•. *See* Phenoxy radical intermediate (PhenO•)
Phenoxy radical intermediate (PhenO•), 119
Phenylalanine ammonia lyase (PAL), 101
Phorbol myristate acetate, 290
Phytochemicals, 16
PK. *See* Protein kinase (PK)
PKC. *See* Protein kinase C (PKC)
PLA. *See* Polylactide (PLA)
Pollution, 3
Polyethylene glycol (PEG), 241
Polylactide (PLA), 242
Polyphenol antioxidants
  activity in *Escherichia coli*, 129
  disease prevention studies, 106
  human cells, 139
  inflammatory markers and signal transaction pathways, 141*t*
  inhibit oxidative stress
    enzyme regulation, 130
    metal binding, 134
    ROS and RNS scavenging, 130
  mechanisms in cells, 118
  oxidative stress, 100
  potential binding sites, 123*f*
  prooxidants, 136
  reduction potential, 144*t*
  RNS scavenging, 101
  ROS scavenging, 100
  structure, 111*f*
  structures and sources, 101
  supplementation in animal studies and clinical trials, 107*t*
Polyphenol compounds as prooxidants, 136
  mechanisms in *E. coli*, 138*f*
Polyphenol enzyme regulation, human cells, 146
Polyphenol-metal chelation, human cells, 148
Polyunsaturated fatty acids (PUFA), 26, 256
PON. *See* Peroxynitrite (PON)
Post-translational modifications (PTM), 370, 383
PQ-mediated ROS generation, 227
Pregnancy, 26
  micronutrient supplementation, 59
Protein complexes, 343
Protein kinase (PK), 42
Protein kinase C (PKC), 146
Protein-protein interactions, 343
  BN-PAGE and WB as a tool for identification of transient, 351
  determination of transient, 345
  possible theoretical scenarios for the analysis, 352*f*
Proteins, 9
Protein sulfhydryl groups, protection, 79*f*
Proteomic analysis
  mass spectrometry, 370
  schematic workflow, 371*f*

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

Pseudomonas aeruginosa azurin, 297
PTM. *See* Post-translational modifications (PTM)
PUFA. *See* Polyunsaturated fatty acids (PUFA)
Pulmonary disorders, 9
Pycnogenol, 123
pY99-IP. *See* Anti-phosphotyrosine (pY99-IP)

Q

QM. *See* Quantum mechanical (QM)
Quantum mechanical (QM), 194
Quantum mechanical calculations, 182
Quantum Prep Plasmid Miniprep Kit, 181
Quercetin, 120
Quercetin-induced cytotoxicity, 137*f*
Quercetin-3-rhamnoglucoside (rutin), 102
Quinone compounds, 84
Quinone reductases, 93

R

Radiation, 3
Radiation-induced fibrosarcoma (RIF-1), 80
Radical scavengers, reduction potential, 144*t*
Reactive nitrogen species (RNS), 1, 79
  scavenging mechanisms, 119
Reactive oxygen species (ROS), 1, 40, 99, 117
  reactions, 117*f*
  scavenging mechanisms, 119
Receptor tyrosine kinases (RTK), 345
Redox balance, 4
Redox buffering, 79
Redox reactions, equations, 73
Redox state, 240
Renal disorders, 24
Resveratrol, 93, 95, 120
Reye's syndrome, 52
Rheumatoid arthritis, 21
RIF-1. *See* Radiation-induced fibrosarcoma (RIF-1)
RNS. *See* Reactive nitrogen species (RNS)
ROS. *See* Reactive oxygen species (ROS)
ROS/RNS release system, 4
RTK. *See* Receptor tyrosine kinases (RTK)
rutin. *See* Quercetin-3-rhamnoglucoside (rutin)

S

SAM. *See* Self-assembled monolayer (SAM)
San Francisco Men's Health Study (SFMHS), 56
Scanning Electrochemical Microscopy (SECM), 299
SDS-PAGE. *See* Sodium dodecyl-sulfate polyacrylamide electrophoresis (SDS-PAGE)
SECM. *See* Scanning Electrochemical Microscopy (SECM)
Selenium, 11
Self-assembled monolayer (SAM), 217
Semiquinone radical, electronic structure, 195*f*
SENCAR mice, 88
SFMHS. *See* San Francisco Men's Health Study (SFMHS)
S-Glutathiolated protein, 79
Sigma-Aldrich Chemical Company, 214
Small HSP proteins, 392
SOD. *See* Superoxide dismutase (SOD)
Sodium dodecyl-sulfate polyacrylamide electrophoresis (SDS-PAGE), 342, 372
Sodium salt (ctDNA), 214
SoxRS stimulon, 128
  ROS and RNS regulation, 130*f*
  selected genes induced, 129*t*
Sprague-Dawley rats, 121
STAIR. *See* Stroke Therapy Academic Industry Roundtable (STAIR)
Streptococcus gordonii, 301
Stress, 3
Stroke. *See* Cerebral ischemia
Stroke Therapy Academic Industry Roundtable (STAIR), 263
Superoxide, 126
  analytical tools, 291
  biosensors, 291, 292*t*
  cellular level, 304
  direct electrochemical detection, 292
  dismutase-modified electrodes, 297
  electrochemical sensors, 291, 292*t*, 293*f*
  half-life, 290
  living cell detection, 290
  mediated electrochemical detection, 295
Superoxide dismutase (SOD), 5, 116, 120, 256
Superoxide regulation, 128
Surface charge/chemistry/coating, 240
Synthetic antioxidants, 17
Synthetic polyphenols, chemical structure, 114*f*

Downloaded by 89.163.35.42 on June 3, 2012 | http://pubs.acs.org
Publication Date (Web): November 17, 2011 | doi: 10.1021/bk-2011-1083.ix002

T

TBA. *See* Thiobarbituric acid (TBA)
TBARS. *See* Thiobarbituric acid reactive substances (TBARS)
TBHQ. *See* Tertiary butyl hydroquinone (TBHQ)
TDF. *See* Tumor Differentiation Factor (TDF)
TDR. *See* Thioredoxin reductase (TDR)
TEAC. *See* Trolox equivalent antioxidant activity (TEAC)
Tertiary butyl hydroquinone (TBHQ), 17
Testosterone, 85
TGSH. *See* Total glutathione (TGSH)
Thermodynamics
  free radical reactions, 72
Thiobarbituric acid reactive substances (TBARS), 62
Thiobarbituric acid (TBA), 145
Thioredoxin, 77
Thioredoxin reductase (TDR), 45
Thioredoxin system, 75, 78
T24 human bladder cells, 299
T lymphocyte, 20
TNF. *See* Tumor necrosis factor (TNF)
Torrent, 312
Total glutathione (TGSH), 62
Toxins, 4
TPA. *See* 12-O-Tetradecanoylphorbol-13-acetate (TPA)
TPP. *See* Tripolyphosphate (TPP)
Traditional medicinal plants, antioxidant activity, 13*t*
Tripolyphosphate (TPP), 242
TRIS-HC1. *See* Trizma hydrochloride (TRIS-HCl)
Trizma hydrochloride (TRIS-HCl), 214
Trolox equivalent antioxidant activity (TEAC), 243
Trolox equivalent antioxidant capacity assay (TEAC), 119
Tumor Differentiation Factor (TDF), 393
Tumor necrosis factor (TNF), 20
Two-electron reduction potentials, 76*t*
Tyrosine nitration, 314

U

Ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS), 90
UPLC-MS/MS. *See* Ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS)
Urine samples, men, levels of estrogen-DNA adducts, 92*f*
UV-Vis spectroscopy, 241

V

Valosin-containing protein, 348
Vascular cell adhesion protein 1 (VCAM1), 113
Vascular endothelial growth factor (VEGF), 114
VCAM1. *See* Vascular cell adhesion protein 1 (VCAM1)
VE. *See* Vitelline envelope (VE)
VEGF. *See* Vascular endothelial growth factor (VEGF)
Vimang, supplementation, 60
Vitamin E, 5
Vitamins C, 11
Vitamins E, 11
Vitelline envelope (VE), 353
Voltammetry, linear potential scan, 219*f*, 220*f*

W

Wistar rats, 112
Wound-toleukocyte signaling, 290

X

Xanthine oxidase, 257
Xanthine oxidase inhibitors, 17
XEDS. *See* X-ray energy dispersive spectroscopy (XEDS)
XPS. *See* X-ray photoelectron spectroscopy (XPS)
X-ray energy dispersive spectroscopy (XEDS), 327
X-ray photoelectron spectroscopy (XPS), 241

Z

Zona pellucida (ZP), 353
ZP. *See* Zona pellucida (ZP)